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**(34) Title: MENINGOCOCCAL CLASS I OUTER-MEMBRANE PROTEIN VACCINE**

*Outer-membrane vesicles*, Class I outer-membrane proteins of *Neisseria meningitidis*, fragments or oligopeptides containing epitopes of the Class I OMV can be used to immunize against meningococcal disease.

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MEMINGOCOCCAL CLASS 1 OUTER-MEMBRANE PROTEIN VACCINEDescriptionBackground of the invention

Bacterial meningitis is an inflammatory disease of the central nervous system caused by the growth of bacteria in and adjacent to the leptomeninges. Meningitis is an acute infectious disease which affects children and young adults and is caused by the *Neisseria meningitidis*, amongst other agents including other bacterial and viral pathogens.

Meningococci are subdivided into serological groups depending on the presence of either capsular or cell wall antigens. Currently recognized serogroups include A, B, C, D, W-135, X, Y, Z, and 98K as segregated by seroagglutination. The polysaccharides responsible for the serogroup specificity of the group A, B, C, X, W-135 and Y have been purified.

The carrier rate for meningococci is much higher than the incidence of the disease. Some persons are temporary carriers, while others are chronic carriers, discharging meningococci either more or less continuously or in a sporadic fashion. The meningococcal carrier state is an immunizing process, and within two weeks of colonization, production of antibodies to meningococci can be identified. It appears that bactericidal antibodies are directed against both the capsular polysaccharide and other cell wall antigens.

Studies have shown that meningococcal outer membranes have three to five major proteins, with the predominant 41,000Mr or 38,000Mr proteins carrying the serotype specific determinants. There is a considerable degree of interstrain heterogeneity in the profiles of the outer membrane proteins on sodium dodecyl sulfate-polyacrylamide electrophoretic gels (SDS-PAGE). As defined by peptide mapping studies, the proteins comprise five classes, designated 1 through 5, based upon common peptide structures. Bactericidal monoclonal antibodies have been produced against the 46,000 Mr Class 1 proteins which are shared to some extent among strains of different serotypes. (Prasch, C.E. et al., (1985) pg. 633, "New Developments in Meningococcal Vaccines", in G.K. Schoolnik et al. (ed.) The Pathogenic *Neisserias*, American Society for Microbiology, Washington, D.C.).

The capsular polysaccharide of groups A, C, W-135 and Y meningococci have been used to develop vaccines against the organism. Although these vaccines have been effective in the short term, they do not induce immunological memory and subjects must be revaccinated within approximately 3 years to maintain their resistance. The group B polysaccharide is poorly immunogenic and successful vaccines have not been produced. A possible explanation for the low activity may be due to tolerance to the group B polysaccharide induced by crossreactive

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antigens found in human tissues such as the brain. Furthermore, studies show that most of the bactericidal antibodies in the convalescent sera of patients who have had Group B meningococcal disease are directed against outer membrane proteins.

Vaccines for protecting against Group B meningococcal disease have been developed in which non-covalent complexes of outer membrane proteins (OMP) and group B polysaccharide were administered. Bouvery, et al. (1983) Infect. Immun. 50:369-380. However, the B polysaccharide is known to induce a transient IgM antibody response, which does not confer immunoprotection. Furthermore, there is great antigenic diversity and variability in the meningococcal outer membrane proteins from strain to strain. Additionally, lipopolysaccharides are present in the OMP and exhibit antigenic variability as well.

There is a need for safe and effective vaccines against meningococcal disease which provide immunity from infection, particularly in infants and the elderly.

#### Summary of the Invention

This invention pertains to isolated outer membrane vesicles (OMV's), to substantially purified Class I outer membrane protein (OMP) of Neisseria meningitidis, to fragments of the Class I OMP and to oligopeptides derived from the Class I OMP which contain continuous or discontinuous, immunogenic and

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protective B cell epitopes reactive with bactericidal antibodies against N. meningitidis and to the use of isolated OMV's, the meningococcal Class I OMP, fragments or oligopeptides for vaccination against N. meningitidis.

The isolated OMV's, meningococcal Class I OMP, fragments, or oligopeptides derived therefrom can be used in univalent or multivalent subunit vaccines alone, in mixtures, or as chemical conjugates or genetic fusions. In preferred vaccines, epitopes from different epidemiologically relevant meningococcal strains are used. In addition, isolated OMV's, the Class I OMP, fragments or oligopeptides can be used in conjunction (as mixtures, fusion or conjugates) with other antigens of N. meningitidis. For example, they can be used in conjunction with capsular polymers or oligomers (or fragments thereof) of N. meningitidis or with Class I outer membrane proteins (or epitopes thereof) of different subtypes. In addition, they can be used with antigens of other infectious bacteria, viruses, fungi or parasites. Class I OMP or cell epitopes also are defined and these can be used in conjunction with other vaccine components to enhance the protective immune response to the vaccines.

This invention also pertains to the methods of producing isolated OMV's, the Class I OMP, fragments and oligopeptides and to various vaccine formulations containing them. The isolated OMV's Class I OMP can be produced by mutant meningococcal strains which do not express the Class 2/3

outer membrane protein. Fragments can be produced by cyanogen bromide cleavage and subsequent purification. Isolated ONV's, the Class I OMP, fragments or oligopeptides can be produced by recombinant DNA techniques, chemical synthesis or chemical or enzymatic cleavage. These materials, in turn, can be conjugated or fused to carrier peptides or proteins, to other antigens of *N. meningitidis* or to antigens of other microorganisms by chemical or genetic coupling techniques to produce multivalent antigenic conjugates and fusion peptides or proteins. They can be modified for conjugation such as by the addition of amino acids or other coupling groups. For vaccination, isolated ONV's, the class I OMP, fragments or oligopeptides, in any of the forms described, can be formulated in pharmaceutically acceptable vehicles with optional additives such as adjuvants.

This invention also pertains to isolated nucleic acids which encode class I OMP, fragments or oligopeptides. The nucleic acids can be incorporated into appropriate expression systems for production of isolated ONV's, Class I OMP, fragments or any oligopeptides derived therefrom. These nucleic acids can be modified as genetic fusions to contain sequences encoding additional polypeptides useful in enhancing the immune response to the vaccine formulation containing the expressed fusion polypeptides. In addition, Class I OMP of *N. meningitidis* is homologous in amino acid sequence and structure to porin proteins of other gram negative pathogens and

thus the Class I OMP, fragments and oligopeptides of this invention allow for the development of vaccines for other gram negative pathogens.

#### Brief Description of the Figures

Figure 1. Scheme for amplification of genes encoding meningococcal Class I outer membrane protein by PCR (Polymerase Chain Reaction).

Figure 2. 5' gene sequences encoding VR1 (first variable region) of Class I outer membrane proteins of several *N. meningitidis* subtypes.

Figure 3. 3' gene sequences encoding VR2 (second variable region) of Class I outer membrane proteins of several *N. meningitidis* subtypes.

Figure 4. Epitope scanning by reaction of monoclonal antibodies with solid phase decapeptides spanning the predicted amino acid sequences of Class I proteins from strains Pl.7,16, Pl.16 and Pl.15.

Adjacent decapeptides differ by five amino residues. Annotations show the strain from which the sequence was derived, the mAb used and its subtype specificity.

Figure 5. Reaction of the monoclonal antibodies with series of overlapping decapeptides corresponding to variable regions VR1 and VR2, with adjacent peptides differing by a single amino acid residue. Annotations show the strain from which the sequence was derived, the mAb used and its subtype specificity.

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Figure 6. Construction of recombinant flagellins expressing variable region epitopes of *N. meningitidis* Class I OMP subtype Pl.6.16.

Figure 7. Structure of recombinant flagellins expressing variable region epitopes of *N. meningitidis* Class I OMP subtype Pl.6.16.

Figure 8. Representative chromatogram of high performance liquid chromatography of a recombinant flagellin.

Figure 9. Representative analysis by SDS-PAGE of recombinant flagellin.

Figure 10. Representative Western blot analysis of a conjugate comprising an epitope of *N. meningitidis* Class I OMP conjugated to CNM 197.

Figure 11. Putative conformation of *N. meningitidis* Class I OMP subtype Pl.16.

#### Detailed Description of the Invention

This invention pertains to vaccines comprising isolated OMV's, meningococcal Class I OMP, fragments of the OMP (e.g., prepared by the application of cyanogen bromide) and oligopeptides bearing epitopes of the OMP; the preparation of isolated OMV's, pure Class I outer-membrane proteins, using mutant strains which do not express the Class 2/3 outer-membrane protein; the preparation of isolated OMV's pure Class I outer-membrane proteins with the aid of cloned DNA in recombinant DNA expression vectors. This invention also comprises the application of genetic engineering with the object of producing isolated OMV's Class I OMP or portions thereof.

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Genetic fusions of Class I OMP, portions or epitopes thereof; and the preparation of multivalent Class I outer-membrane vaccine through peptide synthesis, as the epitopes with a short peptide chain can be synthetically prepared.

It has emerged that meningococcal Class I outer-membrane proteins induce a strong bactericidal immune response to the strains containing the appropriate subtype epitopes, irrespective of whether these are from group A,B,C,Y-135, and Y strains. The polysaccharide vaccine can be enhanced or replaced by a vaccine according to the invention as a vaccine with broad, extensive action against most serotypes. The protective bactericidal monoclonal antibodies specific for the Class I outer-membrane protein react strongly with fragments that have been split off and short synthetic peptides which have been prepared using the amino acid sequence of Class I outer-membrane proteins. Since meningococcal disease is currently caused chiefly by group B meningococci and because the Class I outer-membrane proteins occurring in group B meningococci also occur in group A,C,Y-135,Y meningococci, vaccines of this invention which comprise one or more Class I OMP epitopes derived from *N. meningitidis* Group B should be effective in preventing disease caused by group A, C, Y-135 and Y. Preferably, the preparation of such a vaccine starts from at least two different immunogenic and protective epitopes which have been selected on

epidemiological grounds. Vaccines according to the invention comprise, for example, at least one protein which is obtained either in OMV formulation or by purification from mutant strains producing one or more Class I OMP or at least two fragments prepared through a cyanogen bromide fragmentation or at least two synthetic peptides, chosen from about 10 major epitopes, or products obtained by gene expression via recombinant DNA technology, which contain the desired epitopes. To maximize efficacy to a broad range of meningococcal strains, the greater number of different protective epitopes in the vaccine the better. In addition, the vaccines according to the invention may advantageously contain meningococci A and C or optionally W-135 and Y polysaccharides and/or detergents. Preferably, the A and C polysaccharides are covalently coupled to a protein or polypeptide carrier. These carriers include, for example, isolated OMV, the Class I OMP protein, I-helper epitopes, bacterial toxins, toxoids, nontoxic mutants (CRM's), recombinant *Salmonella* flagellin and viral particles such as rotavirus VP6 protein, Hepatitis B surface antigen or parvovirus VP1 and VP2 proteins. Both Zwitterionic, cationogenic, anionogenic and nonionogenic detergents can be used. Examples of such detergents are Zwittergent 3-10, Zwittergent 3-14 (N-tetradecyl-N, N-dimethyl-3-ammonia-1-propane sulphate), Tween-20, sodium deoxycholate, sodium cholate and octylglucoside. The vaccines according to the invention may also contain an adsorbent such

as aluminium hydroxide, calcium phosphate; or advantageously, aluminium phosphate. The fragments, proteins, peptides can also be processed in immunostimulating complexes (ISCOMs), liposomes or microspheres for delivering and/or use as an adjuvant or in connection with other adjuvants so that greater immunogenicity is obtained.

This invention encompasses isolated OMV, substantially pure meningococcal class I outer membrane proteins (of any subtype) and fragments of the proteins containing epitopes thereof. The fragments can be any portions of the molecular weight of 23kd or less which contain epitopes which are bound by protective bactericidal antibodies against *N. meningitidis*. These include proteolytic fragments and synthetic oligopeptides which are comprised of amino acid sequences which correspond, at least in part, to epitopes of a Class I OMP.

The isolated OMV's, Class I OMP, fragments or epitope-containing oligopeptides derived therefrom can be comprised of amino acid sequences which are different, but essentially biologically equivalent to the natural sequences. These sequences can include sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration.

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Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include glycine, alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include serine, threonine, cysteine, cytosine, asparagine, and glutamine. The charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic and glutamic acids.

Additionally, isolated ONV's, the class I ONP, fragments of the oligopeptides can be modified for conjugation to other molecules (e.g., by the attachment of coupling groups such as the amino acids cysteine and/or lysine or other linking groups and/or spacer groups) including other class I ONP of a different subtype, T cell epitopes, B cell epitopes, carrier peptides or proteins or adjuvanting molecules.

As described in detail below, the Class I ONP, fragments or oligopeptides can be used in many different forms (e.g., alone, in mixtures, or as conjugates and genetic fusions produced from recombinant DNA vectors) in vaccines. For those purposes, the materials can be produced by isolation from *N. meningitidis*, by proteolytic digestion, by chemical synthesis, or by expression as recombinant molecules. The methods of production and use of the isolated ONV's, the class I ONP and the fragments and the oligopeptides of class I ONP are described below.

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Protein modeling and structure analysis of the Class I ONPs were performed using the principles for several *E. coli* outer membrane proteins. (Vogel, H. et al., *J. Mol. Biol.*, 190:191 (1986); Perence, T. et al., *J. Mol. Biol.*, 201:493 (1988) and Tomassen, J. in "Membrane Biogenesis", NATO ASI Series B16, pp351, Springer-Verlag, NY (1988)). The derived amino acid sequence of the Class I ONPs were used for the modeling studies and comparison. The amino acid sequence homology was compared to other gram negative bacterial porin proteins and similarity was established for the protein structure. Exposed surface loops and transmembrane structure were very similar for these porin proteins. With the information revealed concerning variable and constant region protective epitopes of *N. meningitidis* and their structure, one can predict based upon the amino acid sequence where protective epitopes may reside for other pathogenic gram negative bacteria to be evaluated and included in vaccines for the same.

#### Production of Isolated ONV's

ONV's can be produced either from the culture supernatant or from the bacterial cells after fragmentation as described by Bauvery et al. (1983) *loc. cit.* ONV's carrying proteins from more than one meningococcus can be isolated from strains manipulated to express heterologous proteins.

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Production and Purification of Class I OMP and CNBr fragments thereof

Class 1 and Class 3 outer membrane proteins can be isolated as described by Beuvery, E.C. *et al.*, Antonie van Leeuwenhoek J. Microbiol. **52**:232 (1986). The production of substantially pure Class I OMP free of Class 2 or 3 OMP's is achieved by this method using mutant meningococcal strains which do not express Class 2/3 OMP. A preferred strain for production of Class I OMP is the H115 strain, deposited as CBS 636.89.

Fragments can be produced by cyanogen bromide cleavage as described by Tearlink *et al.*, *J. Exp. Med.* **165**:63 (1987) for a gonococcal protein. The N-terminal fragment is referred to as CB-1 and the C-terminal fragment is referred to as CB-2. These CNBr fragments can be purified via reverse phase HPLC on a Vydac C4 or an Aquapore R-300 column using a water/acetonitrile gradient. Alternatively, the fragment can be purified by multiple cold trichloroacetic acid precipitations. These procedures remove greater than 95% of interfering contaminants (e.g., buffer salts, detergents and fragment contaminants).

Preparation of fragments and oligopeptides containing epitopes of class I OMP

A. Preparation by proteolytic digestion

Oligopeptides containing epitopes reactive with bactericidal antibodies against *N. meningitidis* can be produced by digestion of the class I OMP, CB-1 or CB-2 fragments with proteinases such as endolys-C,

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endoArg-C, endoGlu-C and staphylococins V8-protease. The digested fragments can be purified by, for example, high performance liquid chromatographic (HPLC) techniques.

B. Preparation by Chemical synthesis

Oligopeptides of this invention can be synthesized by standard solid peptide synthesis (Barany, G. and Merrifield, R.B., The Peptides **2**:1-284, Gross, E. and Meienhofer, J., Eds., Academic Press, New York) using tart-butylloxycarbonyl amino acids and phenylacetamidomethyl resins (Mitchell, A. R. *et al.*, J. Org. Chem. **43**:2845-2852 (1978)) or 9-fluorenylmethyloxycarbonyl amino acids on a polyamide support (Dryland, A. and Sheppard, R.C., J. Chem. Soc. Perkin Trans. I, 125-137 (1986)). Alternatively, synthetic peptides can be prepared by peptidyl synthesis (Gayson, H.M. *et al.*, J. Immunol. Methods **93**:259 (1987); Proc. Natl. Acad. Sci. USA **81**:3998 (1984)), Cambridge Research Biochemicals, Cambridge, U.K. or by standard liquid phase peptide synthesis. The deletion or substitution of amino acids (and including extensions and additions to amino acids) in other ways which do not substantially detract from the immunological properties of the oligopeptide.

C. Preparation by recombinant DNA techniques

The Class I OMP, fragments and oligopeptides which exhibit epitopes of the Class I OMP can be



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produced by recombinant DNA techniques. In general, these entail obtaining DNA sequences which encode the desired OMP. (Barlow *et al.*, (1989) *Mol. Micro.* 3:131) fragment or oligopeptide sequences and introducing into an appropriate vector/host expression system one or more similar or different DNA sequences of Class I OMP's where it is expressed. The DNA can consist of the gene encoding the Class I OMP or any segment of the gene which encodes a functional epitope of the OMP. The DNA can be fused to DNA encoding other antigens of *E. Meningitidis* (such as other outer membrane proteins either of the same or different class) or antigens of other bacteria, viruses, parasites or fungi to create genetically fused (sharing a common peptide backbone), multivalent antigens. For example, Class I OMP fragments can be fused to another class I outer membrane protein of a different subtype (or fragments or epitopes thereof) of *E. Meningitidis* to yield fusion proteins comprising multiple class I outer membrane protein subtype determinants.

Genetic engineering techniques can also be used to characterize, modify and/or adapt the encoded peptides or proteins. For example, site directed mutagenesis to modify an OMP fragment in regions outside the protective domains, for example, to increase the solubility of the subfragment to allow easier purification. DNA can also be manipulated to effect superproduction of OMP fragments or combinations thereof in various organisms.

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DNA encoding a Class I OMP, fragments or oligopeptides can be synthesized or isolated and sequenced as described by Barlow, A.K. *et al.* *Immun.* 55:2734-40 (1987) and Barlow, A.K. *et al.* *Mol. Micro.* 3:131 (1989). Class I OMP genes can be amplified from bacterial DNA by the methods of Mullis and Faloona, (1987) *Method. Enzym.* 155:335-350, using the primer sequences disclosed herein. Related DNA sequences for class I OMP of different subtypes can be obtained by the procedures described and the amino acid sequences deduced.

A variety of host-vector systems can be used to express the oligopeptides of this invention. Primarily the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus). The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

In order to obtain efficient expression of the cloned DNA, a promoter must be present in the expression vector. RNA polymerase normally binds to the promoter and initiates transcription of a gene

or a group of linked genes and regulatory elements (called an operon). Promoters vary in their "strength", i.e., their ability to promote transcription. It is desirable to use strong promoters in order to obtain a high level of transcription and, hence, a high level of DNA expression. Depending upon the host cell system any one of a number of suitable promoters can be used. For instance, for *E. coli*, its bacteriophages or plasmids, promoters such as the lac promoter, trp promoter, lacA promoter, ribosomal RNA promoter, and  $P_R$  or  $P_L$  promoters of coliphage lambda and others including but not limited to lacUV5, ompF, bla, lpp and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid trp-lacUV5 (tac) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted DNA.

Bacterial host cells and expression vectors may be chosen which inhibit the action of the promoter unless specifically induced. In certain operons the addition of specific inducers is necessary for efficient transcription of the inserted DNA; for example, the lac operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as trp, etc., are under different controls. The trp operon is induced when tryptophan is absent in the growth media; and the  $P_L$  promoter of lambda can be induced by an

increase in temperature in host cells containing a temperature sensitive lambda repressor, e.g., ci857. In this way, greater than 95% of the promoter-directed transcription may be inhibited in unduced cells. Thus, expression of the recombinant peptide or protein can be controlled. This is important if the expression product of the DNA is lethal or detrimental to the host cells. In such cases, transformants may be cultured under conditions such that the promoter is not induced; then, when the cells reach a suitable density in the growth medium, the promoter can be induced for production of the protein.

One such promoter/operator system is the "tac" or trp-lac promoter/operator system (Russell and Bennett, 1982, Gene 20:2312-243; DeBoer, European Patent Application, 67, 540 filed May 18, 1982). This hybrid promoter is constructed by combining the -35 b.p. (-35 region) of the trp promoter and the -10 b.p. (-10 region or Pribnow box) of the lac promoter (the sequences of DNA which are the RNA polymerase binding site). In addition to maintaining the strong promoter characteristics of the tryptophan promoter, lac is also controlled by the lac repressor.

When cloning in a eukaryotic host cell, enhancer sequences (e.g., the 72 bp tandem repeat of SV40 DNA or the retroviral long terminal repeats or LTRs, etc.) may be inserted to increase transcriptional efficiency. Enhancer sequences are a set of

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eucaryotic DNA elements that appear to increase transcriptional efficiency in a manner relatively independent of their position and orientation with respect to a nearby gene. Unlike the classic promoter elements (e.g., the polymerase binding site and the Goldberg-Hogness "TATA" box) which must be located immediately 5' to the gene, enhancer sequences have a remarkable ability to function upstream from, within, or downstream from eucaryotic genes; therefore, the position of the enhancer sequence with respect to the inserted DNA is less critical.

Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires a Shine-Dalgarno (SD) sequence about 7-9 bases 5' to the initiation codon (ATG) to provide a ribosome binding site. Thus, any SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *gfc* gene or the *N* gene of coliphage lambda, or from the *E. coli* cryptophan E, D, C, B or A genes.

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Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Any of the methods described for the insertion of DNA into an expression vector can be used to ligate a promoter and other genetic control elements into specific sites within the vector. *N. meningitidis* sequences for expression can be ligated into an expression vector at a specific site in relation to the vector promoter and control elements so that when the recombinant DNA molecule is introduced into a host cell the foreign genetic sequence can be expressed (i.e., transcribed and translated) by the host cell.

The recombinant DNA vector can be introduced into appropriate host cells (bacteria, virus, yeast, mammalian cells or the like) by transformation, transduction or transfection (depending upon the vector/host cell system). Host cells containing the vector are selected based upon the expression of one or more appropriate gene markers normally present in the vector, such as ampicillin resistance or tetracycline resistance in pBR322, or thymidine kinase activity in eucaryotic host systems. Expression vectors may be derived from cloning vectors, which usually contain a marker function. Such cloning vectors may include, but are not limited to the following: SV40 and adenovirus, vaccinia virus vectors, insect viruses such as baculoviruses, yeast vector, bacteriophage vectors such as lambda gt-10S-

lambda B, Charon 28, Charon 4A, lambda gt-1-lambda BC, lambda gt-1-lambda B, M13mp7, M13mp8, M13mp9, or plasmid DNA vectors such as pBR322, pAC105, pVA31, pACYC177, pMK47, pACYC184, pUB110, pMB9, pBR325, Col E1, pSC101, pBR113, pML21, RSP2124, pCR1, RP4, pBR328 and the like.

Expression vectors containing the DNA inserts can be identified by three general approaches: (1) DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted gene; (2) presence or absence of "marker" gene functions (e.g., resistance to antibiotics, transformation phenotype, thymidine kinase activity, etc.); and (3) expression of inserted sequences based on the physical immunological or functional properties of the gene product.

Once a putative recombinant clone which expresses a desired Class I OMP amino acid sequence is identified, the gene product can be analyzed as follows. Immunological analysis is especially important because the ultimate goal is to use the gene products in vaccine formulations and/or as antigens in diagnostic immunoassays. The expressed peptide or protein should be immunoreactive with bactericidal antibodies against *E. meningitidis*. This reactivity may be demonstrated by standard immunological techniques, such as radioimmuno-precipitation, radioimmuno competition, ELISA or immunoblots.

Once the gene product is identified as a Class I OMP fragment or an oligopeptide containing a functional epitope thereof, it can be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and mixing column chromatography), centrifugation, differential solubility, or by any other standard techniques for the purification of proteins. Several techniques exist for purification of heterologous protein from prokaryotic cells. See e.g., Olson, U.S. Patent No. 4,518,526, Weizel, U.S. Patent No. 4,599,197 and Hung et al., U.S. Patent No. 4,734,362. The purified preparation however produced should be substantially free of host toxins which might be harmful to humans. In particular, when expressed in gram negative bacterial host cells such as *E. coli* or *Salmonella*, the purified peptide or protein should be substantially free of endotoxin contamination.

Class I OMP fragments and oligopeptides of this invention can be formulated as univalent and multivalent vaccines. These materials can be used as produced or isolated by the methods described above. They can be mixed, conjugated or fused with other antigens, including B or T cell epitopes of other antigens. In addition, they can be conjugated to a carrier protein as described below for oligopeptides.

When a heptenic oligopeptide is used (i.e., a peptide which reacts with cognate antibodies, but

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cannot itself elicit an immune response), it can be conjugated to an immunogenic carrier molecule.

Conjugation to an immunogenic carrier can render the oligopeptide immunogenic. The conjugation can be performed by standard procedures. Preferred carrier proteins for the heptenic oligopeptides are toxins, toxoids or any mutant crossreactive material (CRM) of the toxin from tetanus, diphtheria, pertussis, Pseudomonas, E. coli, Staphylococcus, and Streptococcus. A particularly preferred carrier is CRM 197 of diphtheria toxin, derived from E. diphtheriae strain C7(p 197) which produces CRM 197 protein. This strain has ATCC accession no. 53281.

Alternatively, a fragment or epitope of the carrier protein or other immunogenic protein can be used. For example, the hapten can be coupled to a T cell epitope of a bacterial toxin, toxoid or CRM. See U.S. Patent Application Serial No. 150,668, filed February 1, 1988, entitled "Synthetic Peptides Representing a T-Cell Epitope as a Carrier Molecule for Conjugate Vaccines", the teachings of which are incorporated herein. Other carriers include viral particles composed of Rotavirus VP6, Hepatitis B surface antigen or parvovirus VP1 and VP2.

The peptides or proteins of this invention can be administered as multivalent subunit vaccines in combination with antigens of N. meningitidis or antigens of other organisms. Some of the other organisms include the pathogenic bacteria N. meningitidis, N. meningitidis, D. catarrhalis, N. meningitidis, N. meningitidis, etc. For example, they may be administered in conjunction with oligo- or polysaccharide capsular components of N. meningitidis. The capsular components can be derived from any of the serological groups, including A, B, C, D, X, Y, Z, 29B and 435.

Class 1 outer membrane proteins of different subtypes can be used. These may be used in combination to evoke bactericidal antibodies against N. meningitidis. For example, a fragment derived from class 1 outer membrane protein of the Pl.7.16 subtype can be used together with outer membrane proteins or fragments of outer membrane proteins of other subtypes, such as Pl.1, Pl.1.16; Pl.2; Pl.6; Pl.9; Pl.15; Pl.16; or Pl.4 (Abdillahi, N. 25 21, 1988 Micro. Pathog. 5:27) or with meningococcal polysaccharides in mixtures or as chemical conjugates. For combined administration with epitopes of other outer membrane proteins, they can be administered separately, as a mixture or as a conjugate or genetic fusion peptide or protein. The conjugates can be formed by standard techniques for coupling proteinaceous materials or techniques for coupling saccharide polymers to proteins. Fusions can be expressed from fused gene constructs prepared by recombinant DNA techniques as described.

As mentioned, Class 1 OMP, fragment or any oligopeptides derived therefrom can be used in conjunction with antigens (e.g., polymer capsules or saccharide units, envelope or surface proteins) of

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other pathogenic organisms (e.g. bacteria (encapsulated or nonencapsulated), viruses, fungi and parasites). Additional examples of other organisms include respiratory syncytial virus, rotavirus, malaria parasites, and Cryptosporidium parvum.

In formulating the vaccine compositions with the peptide or protein, alone or in the various combinations described, the immunogen is adjusted to an appropriate concentration and formulated with any suitable vaccine adjuvant. Suitable adjuvants include, but are not limited to: surface active substances, e.g., hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyl-di-octadecylammonium bromide), methoxyhexadecylglycerol, and pluronic polyols; polyamines, e.g., pyran, dextran sulfate, poly IC, carbopol; peptides, e.g., muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral salts, e.g., aluminum hydroxide, aluminum phosphate, etc., lymphokines and immune stimulating complexes (ISCONS). The immunogen may also be incorporated into liposomes, microspheres, or conjugated to polysaccharides and/or other polymers for use in a vaccine formulation.

The vaccines can be administered to a human or animal in a variety of ways. These include intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal routes of administration.

#### Live vaccines

The peptide and proteins of this invention can be administered as live vaccines. To this end, recombinant microorganisms are prepared that express the peptides or proteins. The vaccine recipient is inoculated with the recombinant microorganism which multiplies in the recipient, expresses the Class I OMP, fragment or oligopeptide thereof and evokes an immune response to N. meningitidis. Live vaccine vectors include: adenovirus, cytomegalovirus and preferably, pox viruses such as vaccinia (Paoletti and Pincelli, U.S. Patent No. 4,603,112) and attenuated Salmonella strains (Stocker, U.S. Patent No. 4,530,081 and Curtiss et al., Vaccine 6:153-160 (1988)). In addition, class I OMP epitopes can be incorporated into the flagella of attenuated bacterial strains.

Live vaccines are particularly advantageous because they lead to a prolonged stimulus which can confer substantially long-lasting immunity. When the immune response is protective against subsequent N. meningitidis infection, the live vaccine itself may be used in a preventative vaccine against N. meningitidis.

Multivalent live vaccines can be prepared from a single or a few recombinant microorganisms that express different epitopes of N. meningitidis (e.g., other outer membrane proteins from other subtypes or epitopes thereof). In addition, epitopes of other

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pathogenic microorganisms can be incorporated into the vaccine. For example, a vaccinia virus can be engineered to contain coding sequences for other epitopes in addition to those of *N. meningitidis*. Such a recombinant virus itself can be used as the immunogen in a multivalent vaccine. Alternatively, a mixture of vaccinia or other viruses, each expressing a different gene encoding for different epitopes of outer membrane proteins of *N. meningitidis* and/or epitopes of other disease causing organisms can be formulated as a multivalent vaccine.

An inactivated virus vaccine may be prepared. Inactivated vaccines are "killed", i.e., infectivity has been destroyed, usually by chemical treatment (e.g., formaldehyde treatment). Ideally, the infectivity of the virus is destroyed without affecting the proteins which carry the immunogenicity of the virus. In order to prepare inactivated vaccines, large quantities of the recombinant virus expressing the desired epitopes are grown in culture to provide the necessary quantity of relevant antigens. A mixture of inactivated viruses expressing different epitopes may be used for the formulation of "multivalent" vaccines. In certain instances, these "multivalent" inactivated vaccines may be preferable to live vaccine formulation because of potential difficulties arising from mutual interference of live viruses administered together. In either case, the inactivated virus or mixture of viruses may be formulated in a suitable adjuvant in

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order to enhance the immunological response to the antigens. Suitable adjuvants include: surface active substances, e.g., hexadecylamine, octadecyl amino acid esters, octadecylamine, lysolecithin, dimethyl-dioctadecylammonium bromide, N, N'-dioctadecyl-N', N''bis (2-hydroxyethyl-propylamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines, e.g., pyran, dextran sulfate, poly IC, carbopol; peptides, e.g., muramyl dipeptide and derivatives thereof, dimethylglycine, tucatin; oil emulsions; and mineral gels, e.g., aluminum hydroxide, aluminum phosphate, and lyphokines.

#### EXEMPLIFICATION

##### EXAMPLE 1: Monoclonal Antibodies Against Class I OMP's and Their Biological Activity

Type specific monoclonal antibodies were prepared against various meningococcal Class I outer-membrane proteins. These monoclonal antibodies recognize the following subtypes: Pl.1; Pl.2; Pl.6; Pl.7; Pl.9; Pl.10; Pl.13; Pl.16; and Pl.17 (now called Pl.14). The monoclonal antibodies are available as "Monoclonal Kit Serotyping Meningococci" from the RIVM, Bilthoven, The Netherlands. All these monoclonal antibodies react with the SDS (sodium dodecyl sulphate) denatured protein when tested by Western blotting. It also emerged that a number of these monoclonal antibodies reacted with a

25kd CNBr fragment of the 42kd Class 1 outer-membrane protein (see below). This result implied that the Class 1 outer-membrane protein epitopes are mainly of linear type and can therefore be copied with synthetic peptides. The epidemiological results of tests carried out by the Applicants show that the described monoclonal antibodies can subtype most of the group A,B,C meningococci which suggests a limited heterogeneity. Each Class 1 outer membrane protein also appears to contain two individual type specific epitopes (Abdillahi and Poolman, Microb. Pathogen, 1988, 4: pages 27-32; idem *PNAS Microbiol. Immunol.* 47: pages 139-144).

The purified Class 1 outer-membrane protein (see below), subtype Pl.7.16, originating from the culture of the Class 2/3 free mutant (N115) appeared to induce a bactericidal antibody response of 1:64 serum dilution in a dose of 2.5 µg in mice. The monoclonal antibodies against meningococci Class 1 outer-membrane proteins, Class 2/3 outer-membrane proteins and lipopolysaccharides were compared as to bactericidal effect. The monoclonal antibodies against the Class 1 outer-membrane proteins appeared to possess the strongest bactericidal activity (see Table 1). The bacterial response was determined as per Poolman, J.T. (1985), in Schoenick, G.J. et al., Eds. 'The Pathogenic Neisseriae' ASM Publications, Washington, D.C., page 562.

TABLE 1

Bactericidal activity of a collection of monoclonal antibodies, directed against the Class 1 (cl 1), Class 2/3 (cl 2/3) and lipopolysaccharide (LPS) of meningococci. (ND = not determined).

Bactericidal activity of antibody pool (titre)			
Test strain	Strain (GP:serotype: subtype:LPS type)	Cl 2/3 pool	Cl 1 pool LPS pool
3006 (B:26:Pl.2:L2)		1000	8000
M981 (B:421.-:L5)		10	ND
M990 (B:6:Pl.6:L/)		10	2000
M978 (B:8:Pl.1:L1.8)		ND	8000
M982 (B:9:Pl.9:L3.7)		500	2000
M355 (B:15:Pl.15:L1.8)		1000	8000
M44/76 (B:15:Pl.7.16:L3.7)		1000	8000
			4000



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The bactericidal activity of these monoclonal antibodies appears to correlate well with the in vivo protective activity as measured in the rat meningitis model of Saukkonen et al., 1987, *Microbiol. Pathogen* 3:261.

EXAMPLE 1A: Construction of meningococcal strains carrying multiple Class 1 genes

Replacement of chromosomal genes by clones, slightly different versions has been described for *Neisseria gonorrhoea*. (Stein, D.C., *Clin. Microbiol. Rev.* 2 (Suppl.), S146-S149 (1989).) We have found that this method can be applied to the Class 1 gene in *Neisseria meningitidis*. This was done in the following way:

(1) The Class 1 gene of strain 2996 (subtype Pl.2) was cloned into the vector pTZ19. (Mead, D.A. et al., *Protein Engineering* 1, 67 (1986).) The complete gene is located on a 2.2 kb XbaI fragment that was ligated to XbaI digested vector DNA.

(11) The resulting plasmid was used for transformation of strain H44/76 (subtype Pl.7.16). Cells of the acceptor strain were incubated with plasmid DNA in the presence of  $Mg^{2+}$  and normal meningococcal medium; they were subsequently diluted and plated, and the

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resulting colonies were tested for their ability to bind Pl.2-specific monoclonal antibody. Such transformants were found with a frequency of approximately  $10^{-5}$ . Further characterization showed that replacement of the H44/76 Class 1 gene had indeed occurred.

An essential feature of the method is the presence of the donor gene on a circular plasmid DNA molecule that is not able to replicate in *N. meningitidis*, since the use of linearized DNA yielded no transformants at all.

Construction of a strain with two Class 1 genes was done by a modification of the method described above. For this purpose, the Pl.2 Class 1 gene was inserted into a clones Class 5 gene. The Class 5 gene family has two features which make it particularly suitable for this construction.

(Meyer, T.Y. and Van Putten, J.P.M., *Clin. Microbiol. Rev.* 2 (Suppl.) S139-S145 (1989): (1) there are four or five Class 5 genes present in the meningococcal genome, and (11) expression of these genes is not necessary for growth under laboratory conditions. A Class 5 gene was cloned from strain H44/76 and the Pl.2 gene was inserted into an SphI site located in or very close to the Class 5 gene. The resulting hybrid plasmid, pHC22, was used for transformation of strain H115, a Class 3-deficient mutant of H44/76. Colonies reacting with the Pl.2-specific monoclonal antibody were isolated and

characterized. Out of 10 such transformants, nine were found to have lost the Pl.16 epitope of the acceptor strain. This indicates that in all these cases recombination has only occurred between the Class 1 genes, resulting in subtype replacement.

However, one transformant was found which made both Class 1 subtypes, i.e., Pl.7.16 and Pl.2, suggesting that recombination between the Class 5 gene sequences on plasmid and chromosome must have occurred. This was confirmed by Western blotting, which revealed the presence of both types of Class 1 protein and by Southern blotting, which demonstrated the acquisition of a second Class 1 gene.

By continuing this construction with other Class 1 subtypes, it is possible to make a strain with four or five different Class 1 genes. The same Class 5 gene can be used in each subsequent transformation step, the different Class 5 genes can be clones and used separately. These recombinant strains can be used to prepare mixtures of different purified Class 1 ONPs.

#### EXAMPLE 12: Purification of isolated ONP's from bacteriological culture

The purification is carried out according to Bauvery et al. (1983) loc. cit.

This culture can be done with the desired wild type strains, mutant meningococci strains without Class 2/3 outer-membrane proteins and/or homologous

and heterologous recombinant microorganisms which express one or more of the desired meningococci Class 1 outer-membrane protein and/or epitopes by overproducing vectors either through or not through existing open reading frames and/or manipulated reading frames so that fusion proteins or proteins with exchanged epitopes can be prepared.

Readily available of wild strains are:

R44/76 (B:15:Pl.7.16) (Holten E., Norway, deposited as CBS 635-89); 187 (B:4:Pl.7) (Etienne J., France); M1080 (B:1:Pl.1.7) (Fraser C., USA); ~~Swiss~~ (B:4:Pl.15) (Hirschel B., Switzerland); B21061 (B:4:Pl.2) (Berger U., West-Germany); 395 (B:NT:Pl.9) (Jonsdottir K., Iceland); M990 (B:6:Pl.6) (Fraser C., USA); 2996 (B:2b:Pl.2) RIVM, The Netherlands; M982 (B:9:Pl.9) (Fraser C., USA); S3446 (B:14:Pl.6) (Fraser C., USA); H355 (B:15:Pl.15) (Holten E., Norway); 6537 (B:17:Pl.17) (Zollinger V., USA) and B40 (A:4:Pl.10) (Achtman M., West-Germany). An example of a Class 3 negative mutant is H115 (B:-:Pl.16) deposited as CBS 636.89.

These strains were inoculated from precultures at -70°C into shake flasks and transferred from these into 40, 150 or 350 litre fermenter cultures. The semisynthetic medium had the following composition: L-glutamic acid 1.3 g/l, L-cysteine.HCl 0.02 g/l, Na<sub>2</sub>HPO<sub>4</sub>.2H<sub>2</sub>O 10 g/l, KCl 0.09 g/l, NaCl 6 g/l, NH<sub>4</sub>Cl 1.25 g/l, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.6 g/l, glucose 5 g/l, Fe(NO<sub>3</sub>)<sub>3</sub> 100 µM, yeast dialysate.

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During culturing in the fermenter, the pH and  $PO_2$  were monitored and automatically regulated to a pH of 7.0-7.2 and an air saturation of 10%. The cells were grown to early stationary phase harvested by means of centrifuging and washing with sterile 0.1 M NaCl and stored at -20°C or freeze-dried.

EXAMPLE 2: Purification of Class 1 outer-membrane proteins from bacteriological culture

This culture can be done with the desired wild type strains, mutant meningococci strains without Class 2/3 outer-membrane proteins and/or homologous and heterologous recombinant microorganisms which express one or more of the desired meningococci Class 1 outer-membrane proteins and/or epitopes by overproducing vectors either through or not through existing open reading frames and/or manipulated reading frames so that fusion proteins or proteins with exchanged epitopes can be prepared.

Readily available of wild strains are:

H44/76 (B:15:PI.7.16) (Holtan E., Norway, deposited as CBS 635-89); 187 (B:4:PI.7) (Etienne J., France); N1080 (B:1:PI.1.7) (Frasch C., USA); 84284 (B:4:PI.15) (Hirschel B., Switzerland); B21061 (B:4:PI.2) (Berger T., West-Germany); 392 (B:HT:PI.9) (Jonasdottir K., Iceland); M990 (B:6:PI.6) (Frasch C., USA); 2996 (B:2b:PI.2) RIVM, The Netherlands; M982 (B:9:PI.9) (Frasch C., USA); 53446 (B:14:PI.6) (Frasch C., USA); H322

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(B:15:PI.15) (Holtan E., Norway); 6557 (B:17:PI.17) (Zollinger W., USA) and B40 (A:4:PI.10) (Achtmann M., West-Germany). An example of a Class 3 negative mutant is H1115 (B:-:PI.16) deposit # CBS 636-89.

These strains were inoculated from precultures at -70°C into shake flasks and transferred from these into 40, 150 or 350 litre fermenter cultures.

The semisynthetic medium had the following composition: L-glutamic acid 1.3 g/l, L-cysteine.HCl 0.02 g/l,  $Na_2HPO_4 \cdot 2H_2O$  10 g/l, KCl 0.09 g/l, NaCl 6 g/l,  $NH_4Cl$  1.25 g/l,  $MgSO_4 \cdot 7H_2O$  0.6 g/l, glucose 5 g/l,  $Fe(NO_3)_3$  100 µM, yeast dialysate.

During culturing in the fermenter, the pH and  $PO_2$  were monitored and automatically regulated to a pH of 7.0-7.2 and an air saturation of 10%. The cells were grown to early stationary phase harvested by means of centrifuging and washing with sterile 0.1 M NaCl and stored at -20°C or freeze-dried.

The bacterial mass was for example extracted with the aid of 0.5 M  $CaCl_2$ , 1% (v/v) Zwittergent 3-14 (Zw 3-14) and 0.14 M NaCl, pH 4.0, using 100 ml per gram of freeze-dried bacterial mass. The suspension was stirred for 1 hour at room temperature and then centrifuged (1 hour, 3000 x g), after which the supernatant was collected in a sterile manner. 20% ethanol (v/v) was added to the supernatant and after stirring for 30 min. the product was centrifuged (30 min., 10,000 x g), after which the supernatant was collected aseptically. The supernatant was then concentrated by means of

diafiltration in an Amicon Hollow Fiber System (HID x 50, cut off 50,000) and  $\text{CaCl}_2$  and ethanol were removed. The concentrate was diluted with 0.1 M sodium acetate, 25 mM EDTA, 0.03% Zw 3-14 having a pH of 6.0 to the original volume and then concentrated again by means of diafiltration. This procedure was repeated five times. The pH of the final concentrate was adjusted to a value of 4.0. 20% (v/v) ethanol was added to the concentrate and, after stirring for 30 min., the product was centrifuged (30 min., 10,000 x g). The whole proteins are purified with the aid of column chromatography in the presence of detergent, for example Zw 3-14. Often gel filtration over Sepharose is as well as the ion exchange over DEAE Sepharose is applied (Neuvery et al., (1986) EMBE). The used extraction method, detergents, column chromatography are not the only applicable method yet only serve as examples and must not be regarded as restrictive.

#### EXAMPLE 3: Preparation and Characterization of Class I OMP Peptide Fragments

Cyanogen bromide was used to prepare fragments of meningococcal Class I outer-membrane proteins. The purified Class I or mixtures of Class I or 3 outer-membrane proteins were taken up in 70% (v/v) formic acid and treated with a 10-fold excess of CNBr for 16 hours at room temperature. The CNBr and the formic acid were removed by means of evaporation

and replaced by 0.2 M Tris-HCl, 6 M urea solution, pH 7.2. The supernatant was prepurified by means of gel filtration over Sepharyl S-200 and subsequently purified with the aid of TSK-2000 gel filtration via RPIC. Neuvery et al., (1986) EMBE.

#### Enzymatic digestion of CB2 fragments

To further delineate the epitopes, the meningococcal CB2 fragment was subjected to digestion with EndoArg-C, EndoClu-C or V-8 and the resulting fragments isolated by HPLC. Briefly, 20 nMoles of CB2 fragment in 1 ml of 25 mM phosphate/0.1 M Tris buffer (pH 8.0) containing 3M urea was digested at 37°C with 0.2 nMoles of EndoArg-C (1 µg/ml in distilled water) or 0.22 nMoles of EndoClu-C or V-8 (1 mg/ml in distilled water) for 14-18 hours. The resulting digested fragments were separated by reverse phase HPLC using a Vydac-C4 column and a trifluoroacetic acid-acetonitrile gradient. The main peak eluted from the EndoArg-C digestion had an apparent molecular weight of 7-9 Kdal while the main peak observed following EndoClu-C or V-8 had an apparent molecular weight of 4-6 Kdals. The isolated peaks were subsequently shown by Western blot to react to a pool of monoclonal antibodies (Adam I, 52-D12-8, MNS-Cl1G and MNI4-Cl16).

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The P1.16 epitope appears to be present on the C-terminal CNBr fragment of the Class I outer-membrane protein of strain B44/76 (3:15; P1.7.16). Further characterization of the P1.16 epitope was carried out through amino acid sequence determination of the 17Kd (N-terminal) and 25Kd (C-terminal) CNBr fragments. The C-terminal 25Kd is further fragmented with V8 protease, endolyso, endoGlu-C and endoArg-C. Fragments which were positive with the P1.16 monoclonal antibody were sequenced as far as possible. The sequences which were obtained are as follows:

**N-terminus of whole protein:**

DVSLYGEIKAGVBNRYQLTEAGDAQR...

**N-terminus of 25Kd C-terminal CNBr**

Fragment : (M) PVSVMYDSEPSGSGSVQFVPIQNS-

KSATPAYTYKDTNR...

Fragments which react with P1.16 monoclonal antibodies were isolated using V8 protease and endoArg-C fragmentation with a molecular weight of 7-9Kd and 4-6Kd respectively. The N-terminal sequences hereof are as follows:

V8 7-9Kd fragment: PSCPSGVQFVPIQNSKSATPAYTYKDTNR...

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Arg-C 4-6Kd fragment: PVSVMYDSEPSGSGSVQFVPI-  
QNSKSATPAYTYK...

**EXAMPLE 4: DNA Sequences of Class I OMP Genes**

Amino acid sequences of Class I OMP were deduced from the nucleotide sequence of the structural genes of four meningococcal Class I OMP's with various subtypes. Comparison with four amino acid sequences enabled a prediction of the composition and the location of these epitopes. Further, the P1.7 and P1.16 epitopes were confirmed with the aid of peptide synthesis and the demonstration of binding of the respective monoclonal antibodies.

Class I OMP genes were cloned into lambda gell (as described for P1.16 in Barlow *et al.*, (1987) *Infect. Immun.* 55: 2743-2740) and subcloned in M13 sequencing vectors and the DNA sequence was determined by standard chain termination dideoxynucleotide techniques.

The complete derived amino acid sequence for P1.16; P1.15, P1.7.16; and P1.2 proteins are as follows:

	10	20	30	40	50
P1.16:	DVSLYGEIKAGVBNRYQLTEAGDAQR	-----	KVTKASRIKTRIS	-----	
P1.15:	DVSLYGEIKAGVBNRYQLTEAGDAQR	-----	KVTKASRIKTRIS	-----	
P1.7.16:	DVSLYGEIKAGVBNRYQLTEAGDAQR	-----	KVTKASRIKTRIS	-----	
P1.2:	DVSLYGEIKAGVBNRYQLTEAGDAQR	-----	KVTKASRIKTRIS	-----	



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EXAMPLE 5: DNA Sequencing of Class I OMP Genes from different N. meningitidis Serogroups

The Polymerase Chain Reaction (PCR) technique of Mullis and Faloona (Methods in Enzymol. 155:335-350, 1987) was used to amplify the entire Class I OMP gene and specific fragments according to the scheme shown in Figure 1.

Primers were synthesized on an Applied Biosystems 380B DNA synthesizer and used in standard PCR 30 cycle amplification reactions using Taq polymerase in a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) according to the recommendations of the supplier. Amplified fragments of about 1300, 900 and 450bp were generated from each serosubtype genomic DNA preparation from the primer combinations shown in Figure 1. The primers used had the following sequences:

PR1: (41 bases with universal primer extension)  
TGT AAA ACC ACG ACC ACG ACT TTT AAG ACG TAT CCG GCG TTT GC

PR2: (42 bases with universal primer extension)  
TGT AAA ACC ACG ACC ACG ACT GGC GAA TTC GGT ACG CTG CCG GCG

PR3: (42 bases with universal primer extension)  
TCT AAA ACC ACG ACC ACG ACT CAT GAG GTA CAC GCG CTG ACG GCG

PR4: (40 bases with universal primer extension)

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TGT AAA ACC ACG ACC GCG ACT GCA GAT TCG CAC TCA CAT TCG A

PR5: (40 bases with universal primer extension)  
TGT AAA ACC ACG ACC GCG ACT GCG ATC GGT ACG TTT GCG TTG A

PR6: (40 bases with universal primer extension)  
TCT AAA ACC ACG ACC ACG ACT AAC TGA TTC GCA ACG CCA CCG G

PRD: (24 bases)  
TTG AAG GAC GTA TCG GGT GTT TCG

REV: (23 bases)  
GCA CAT TCG CAC TCA CAT TCG TT

Excess single stranded template for sequencing was synthesized in an 'asymmetric PCR' amplification using 100x excess of primer carrying an 18 base extension at the 3' end corresponding to the universal fluorescent sequencing primers used with an Model 370A Automated DNA Sequencer (Applied Biosystems, Foster City, CA). Taq polymerase was used in a standard dideoxynucleotide chain termination sequencing reaction with the PCR generated single stranded Class I gene fragments as templates. Derived sequences for gene segments of strains H44/76 (Pl.7.15), M1080 (Pl.1.7), H355 (Pl.15), 6960 (Pl.6), 6557 (Pl.14), 870227 (Pl.10) and 840 (Pl.10) are shown in Figures 2 and 3.

**EXAMPLE 6: Confirmation of Amino Acid Sequences of Class I OMP Subtype Epitopes**

From these gene sequences confirmed by direct sequencing of Class I OMP genes, it was deduced that the sequences corresponding to amino acids 24-34 and 176-187 of Pl.16 are markedly variable in the four Class I OMP sequences. Three amino acid sequences N-terminal or C-terminal from these positions should also be considered for possible inclusion in these epitopes to allow for maximizing epitope stability presentation and unexpected insertions or deletions in the native protein sequence. Further the DNA and amino acid sequences of other Class I OMPs should be compared with the Pl.7.16 sequence to allow for maximum alignment and epitope prediction. The first variable region epitope and second variable region epitope are called VR1 and VR2 respectively. These regions encode the subtype epitopes as was confirmed with the aid of peptide synthesis and the reaction of the peptides with Pl.2; Pl.7; Pl.15 and Pl.16 specific monoclonal antibodies.

A complete set of overlapping decapeptides staggered by 5 amino acids were prepared using the Pl.16 protein sequence. The anti- Pl.16 monoclonal antibody reacted with the decapeptide YTKDTNNL from Pl.16 reacted as expected and no other decapeptide. (Figure 4).

Of overlapping decapeptides provided with a one (1) amino acid sequence shift in the region 24-34

and 176-187 of the Class I OMP of strains H44/76 (Pl.7.16), M250 (Pl.16) and M251 (Pl.15) more than one peptide reacted with the subtype specific monoclonal antibody. In most cases one or more of the group of these overlapping peptides reacted with the subtype specific monoclonal antibody more strongly than others (Figure 5).

These peptides are designated as the VR1 and VR2 epitopes. In the Pl.7.16 strain, the sequence YTKDTNNL is present, the change D to E at residue 180 does have some effect on reducing antibody binding. The sequence HYRQNTDVF in Pl.15 in the same relative position in the protein as the Pl.16 epitope and is responsible for binding to the anti-Pl.15 monoclonal antibody. AQAAGCASC shows some binding and peptides 1-3 amino acids downstream show far greater binding to the Pl.7 monoclonal antibody. Sequence HFVQTPQSQP of VR2 is responsible for binding to the anti-Pl.2 monoclonal antibody. It is probable that the sequences PQVTRGVQCN and PPSKQSP in the Pl.16 and Pl.15 proteins also represent epitopes.

**Example 6B: Class I OMP Constant Region Epitope Identification**

Peptides forming surface loops were prepared and conjugated to tetanus toxoid. A Biolyux 4170 automated peptide synthesizer (Pharmacia/LKB) was used for continuous flow solid-phase synthesis with



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the following exception. In the last cycle of the synthesis SAMA-OTfp (0.5 mmol) (Drijfhout, J. V. (1989), Ph.D. Thesis, Leiden, The Netherlands) was coupled in the presence of 1-hydroxybenzotriazole (0.5 mmol) for 30 min., using a standard protocol with omission of the piperidine-treatment (i.e. the "Proc-deblocking step" which in this case would cause undesirable  $\beta$ -deacetylation). These are referred to as SAMA-peptides.

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The peptides and their surface region location which were conjugated to IT are as follows:

<u>Name</u>	<u>Peptide</u>	<u>Region</u>
LSV 017	176 XGGYTRDTRML	185 P1.16, loop 4
018	24 XGQNMNMQASG	33 P1.7, loop 1
024	276 XGGLSEMGDAKTRSTTE	291 P1.16, loop 6
025a	XGCHAFELFLIGATSDEAKG	245 P1.16, loop 5
025b	223 XAVYGRNAFLFLIGATSDAKG	137 P1.16, loop 5
026	124 XGDSMDVAGQLQIFK	137 P1.16, loop 3
027	XADRTDAENVAVTANAFV	Class 2, loop 5
028a	329 XGCGKKGNTSYDQ	Class 1, loop 7
028b	317 XGDSGKSGNTSYDQ	Class 1, loop 7
029	XGQVDACTTAQCGASRTATQ	Class 2, loop 1
030	78 XQWVVAEGGASQVGN	90 P1.16, loop 2
031	352 XKENTGICNTQINNA	366 P1.16, loop 8
032	16 XGKNIQAQLTQGYVTRGVGN	34 P1.16, loop 1

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Conjugation of SAMA-peptides to tetanus toxoid was performed as follows. A solution of N-succinimidyl bromacetate (4.7 mg, 10  $\mu$ mol) in DMF (100  $\mu$ l) was mixed with a solution of tetanus toxoid (TT) (20 mg) in 0.1 M sodium phosphate buffer pH 7.8 (3.5 ml). After 1h, 1.8 ml of the reaction mixture was subjected to gel filtration using a Sephadex PD-10 column (Pharmacia) equilibrated in 0.1 M sodium phosphate, containing 5mM EDTA (pH 6.1). The bromoacetylated tetanus toxoid was eluted with the same buffer and collected in 3.5 ml. The solution of bromoacetylated tetanus toxoid (1.2 ml) was added to the SAMA peptide (4.5 mg, 3  $\mu$ mol) and desalted with helium. Next, 150  $\mu$ l of 0.2 M hydroxylamine (in PE buffer, pH 6.1) was added. After 16 h remaining bromoacetyl groups were blocked by addition of 2-aminoethanethiol hydrochloride (4  $\mu$ mol) in buffer, pH 6.1 (150  $\mu$ l). After a further period of 16 h, the peptide-TT conjugate was purified by gel filtration over a PD-10 column using PE buffer, pH 6.1, as the eluent. The appropriate fractions were combined and stored at 4°C.

To determine the immunological activity, 25  $\mu$ g (total protein) per dose of a peptide-TT conjugate was injected subcutaneously at weeks 0 and 4 into 6-8 week old NIH outbred mice. (Note: Vaccine L3V 017-TT and L3V 018-TT were used at 10  $\mu$ g total protein/dose.) Sera were collected 6 weeks following the first dose and evaluated for antibody

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response in an ELISA assay (Beuvery, E.C. et al. (1983) Infect. and Immun. 40:3690380). The following antigens were coated into the microtiter wells: Outer membrane protein (OMP), purified Class I OMP (Poolman, J.T. et al. (1989) Infect. and Immun. 57:1005) and the unconjugated peptide. Bactericidal activity (BC) of sera was also measured (Poolman, J.T. et al. (1985) Suppl.)

The results are presented in Table 2 below.

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TABLE 2

<u>Vaccine</u>	<u>OMP</u>	<u>Class 1 OMP</u>	<u>Synth Peptide</u>	<u>Test</u>	<u>Bacterioides</u>
LBV 018-TT	1:900 (0.03)*	1:2700	ND	CI:64	
LBV 017-TT	1:900 (1)	1:900	ND	CI:64	
LBV 024-TT	1:100	1:100	1:900 (homol.)	CI:64	
LBV 023a-TT	-	1:100	1:2700 (homol.)	CI:64	
LBV 023b-TT	1:2700 (4)	1:300	1:8100 (homol.)	CI:64	
LBV 026-TT	-	-	(homol.)	CI:64	
LBV 027-TT	-	1:300	1:300 (homol.)	CI:64	
LBV 028a-TT	1:100	-	1:2700 (homol.)	CI:64	
LBV 028b-TT	1:100	1:100	1:900 (homol.)	CI:64	
LBV 029-TT	-	1:100	1:8100 (homol.)	CI:64	
LBV 030-TT	-	1:100	1:2700 (homol.)	CI:64	
LBV 031-TT	-	1:100	(homol.)	CI:64	
LBV 032-TT	-	1:100	1:900 (homol.)	CI:64	

\* numbers in ( ) indicate O.D. level showing this color

These data suggest that of the constant surface loops tested of Class 1 and 2 OMPs of *N. meningitidis* loop 3 appears to represent at least one region that will produce antibodies which will cross-react with Class 1 and Class 2 OMP of many strains of *N. meningitidis*.

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#### EXAMPLE 7: Construction of recombinant flagellins expressing *N. meningitidis* epitopes

To create hybrid flagella containing epitopes from class I meningococcal epitopes, a series of oligonucleotides was designed based on primary protein sequence data and epitope mapping data. Two oligonucleotides based on VBI or VB2 epitopes of outer membrane P1.7.16 were designed so that they could be cloned in single or multiple copies into a cloning region within the gene for *E. coli* flagellin. Translation termination signals were included on the non-coding strand of the oligonucleotide to facilitate screening by expression of the cloned inserts.

The plasmid vector pPX1650 containing the entire coding region and promoter regions for the structural gene for flagellin H1-d of *Salmonella meninges* (deposited at the ATCC, accession #67685) was modified to contain several unique cloning sites suitable for the insertion of either oligonucleotides or gene fragments in each of the three reading frames of the flagellin gene (Figure 6). First, pPX1650 was digested with *Bco*RV, which cleaves pPX1650 twice, 48 base pairs apart, and religated to yield a plasmid, pPX1651, which has a unique *Bco*RV cloning site and which results in a 16 amino acid deletion in the flagellin protein. pPX1651 was identified by screening *E. coli* recombinants on Western blots probed with polyclonal antibody

directed against H1-d flagellin. pPX1651 was identified amongst several candidates having flagelline smaller than wild type flagellin (of 1650) and was verified by sequencing. Second, pPX1651 was restricted with BamHI and religated after filling out the overhanging ends with Klenow enzyme to remove the unique BamHI restriction enzyme site in the polylinker region of the vector. As a final step, the resulting vector was digested with EcoRV and the following oligonucleotide linker was inserted:

```

5'   ATC ATC GAT GGA TTC   3'
3'   TAG TAG CTA CCT AAG   5'

```

Candidates were screened for the newly created BamHI sites and several candidates having BamHI sites were screened for orientation of the linker by double strand DNA sequencing methodology. One candidate having the linker in the above orientation was retained as pPX1647:

```

5'..... GAT ATC ATC GAT GGA TTC ATC.....
          EcoRV   ClaI   BamHI

```

Plasmid pPX1647 (Figure 7) was digested with BamHI and either oligonucleotides for VR1 or VR2 were cloned into *E. coli* cells. Screening for desired recombinants was accomplished by digesting plasmid miniprep DNA with appropriate diagnostic

restriction enzymes and screening for expression by probing hybrid flagella for decreased mobility on SDS-PAGE gels with specific flagellar antiserum (H1-d). A number of the resultant clones showed decreased mobility on SDS-PAGE, indicating proper insertion of one or more of the oligonucleotides for VR1 or VR2. Several of each were retained for analysis by DNA sequencing. Clone CB1-2 results from tandem insertion of two copies of the VR1 oligonucleotide and clone CB1-4 results from insertion of four oligonucleotides. Likewise CB2 P contained a single insert of the VR2 oligonucleotide and CB2 W showed the expected trimeric insert, CB2 P clone contained a single base pair change which resulted in a change from Leu to Phe in the expressed VR2 fusion protein and was not retained for further study. The recombinant flagellin clones in *E. coli* were probed with monoclonal antibodies (Abdillahi and Poolman, Microbiol. Pathogenesis 4:27-32, 1988; RIVM, The Netherlands) known to react with either VR1 or VR2 epitopes. Monoclonals Adam-1 (P1.7) and Mm14-C11-6 (P1.7) react with hybrid flagellin containing 2 or 4 tandem inserts of VR1, but do not react with clones containing VR2. The weaker reaction of both monoclonals with CB1-2 than with CB1-4 is likely due to epitope density. By the same token, monoclonals 62 (P1.16) and Mm5-c11-G (P1.16) react with CB2 W clone, but not with the VR1 inserts. The CB2 P clone fails to react with either VR2 antibody, probably due to the Leu to Phe change.

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Each of these clones was transformed into an *Escherichia coli* strain (JM109), having a *lacZ* insertion in the *lacZ* locus, to examine the functioning of the hybrid flagella. Each of the four clones resulted in motile bacteria; motility of the transformants was inhibited by the corresponding monoclonal antibody, including clone CB2-7, indicating affinity of the VR2 monoclonal for the epitope in intact flagella. This result indicates that epitopes are exposed at the cell surface and are accessible to antibody.

Hybrid flagellin containing both VR1 and VR2 epitopes were created by cleaving either CB1-2, CB1-4, or CB2-7 with *Bam*HI and cloning the heterologous epitope. Clones CB12-7 and CB12-10 result from the in-frame insertion of a single copy of the VR2 oligonucleotide behind either 2 or 4 VR1 tandem inserts, respectively; clone CB21-F arose from the insertion of one copy of the VR1 epitope behind 3 tandem copies of VR2. CB12-7 and CB12-10 are recognized only by VR1 monoclonal antibody and CB21-F is recognized only by VR2 monoclonal. These results, taken together with DNA analysis revealing predicted sequences, indicate epitope density is too low in the combined hybrids. To create a hybrid flagellin with increased density of both VR1 and VR2 epitopes, CB12-10 was digested with *Bam*HI and VR2 encoding oligonucleotides were inserted. Clone 12-10-6 contains two further tandem inserts of the VR2 epitope, resulting in a hybrid flagellin

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molecule in which four tandem copies of VR1 are followed by three copies of VR2. As is shown in Figure 3a and b, three of the hybrid flagellin vaccine candidates have the expected molecular properties. The flagellin (pCB1 x 4) containing a copy of VR1 reacts with anti-HI-d (anti-flagellin) and anti-VR1 monoclonal antibodies, but not with anti-VR2 monoclonal antibodies; the flagellin (pCB2-V) containing 3 tandem copies of VR2 reacts with anti-HI-d and anti-VR2 antibodies, but not with anti-VR1; the combined hybrid containing copies of VR1 and 3 copies of VR2 reacts with both anti-VR1 and anti-VR2 monoclonal antibodies. The combined hybrid specified motility when introduced into a non-motile recipient *E. coli* strain.

As a subunit vaccine, the goal is to obtain suitable initial vaccine candidates in high quantity and high purity. A suitable vaccine candidate can be chosen from the above type constructions based on reactivity to monoclonal antibodies and function of flagella in non-motile *Salmonella* host strains. A subunit flagellin vaccine may not need to retain all functional aspects of a parental flagellin, but should at least retain surface localization for purification purposes. Several subunit flagellin meningococcal vaccine were chosen from the above described hybrid molecules based on reactivity to monoclonal antibodies and implied surface localization based on restoration of bacterial motility.

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Three flagellin vaccine candidates contained either 4 tandem inserts of VR1, 3 tandem inserts of VR2, or 4 VR1 inserts followed by 3 VR2 inserts. Because flagellin is a major protein of *Salmonella*, it is possible to easily purify sufficient material for vaccination studies using techniques established for flagella purification (Logan *et al.*, J. Bacteriol. 169: 5072-5077, 1987).

EXAMPLE 8: Initial Purification of recombinant flagellin molecules

The three hybrid flagellin vaccine candidates and a wild type (derived from PPK1630) were inoculated into four-liter baffled Fernbach flasks containing 1 liter of LB broth. Bacterial cultures were incubated at 37°C with shaking (200 rpm) for 22-24 hr. Under these conditions of culturing, the bulk of the flagella were sloughed from the bacterial cell surface and were localized in the supernatant culture medium. To obtain suitable material, flagella were isolated from 6-8 liters of culture medium. To obtain purified flagellin preparations for vaccination studies, flagellar filaments were harvested from bacterial culture supernatants by the following procedure: Ammonium sulfate was added to culture supernatant so that final solution was 50% saturated; the solution was stirred gently at 4°C for several hours and the precipitated material was collected by centrifugation in a GSA rotor at 5000

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rpm for 30 minutes. The collected ammonium-sulfate precipitated material was reconstituted in PBS and dialyzed against PBS at 4°C for 12-15 hrs. The dialyzed material was subjected to high speed centrifugation at 100,000 x g for 1 hour in an SW-27 rotor to pellet the flagellar filaments. The pelleted material, which consisted primarily of flagellin, was subjected to further purification by the following method.

EXAMPLE 9: HPLC Purification of recombinant flagellins

To prepare highly purified flagellins, *Salmonella* expressing the constructions, in particular pCH12-10-6, was grown as described above and the cells pelleted at 10,000G. The culture supernatant was then precipitated with 30% ammonium sulfate. The resuspended pellet was dialyzed against 10mM Tris buffer (pH-8.0) containing 6M urea, 1mM PMSF, 2mM NEM, and 5mM EDTA overnight at 4°C. Dialyzed material was then passed over two DEAE sepharose minicolumns (3.0 ml volume, 4.0 ml eluant over each). The columns were eluted (5X) with 50mM NaCl in 10mM Tris (pH-8.0) containing 6M urea and then with 1M NaCl in 10mM Tris (pH-8.0) containing 6M urea. The first four elution collections (20 ml) of the 50mM NaCl were pooled and dialyzed against 1.0 liter 10mM Acetate buffer (pH-4.0) in 6M urea at

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room temperature. The dialyzed fractions were then loaded onto a TSK SP PW cation exchange HPLC column (7.8mm x 300mm). The column was eluted with a mobile phase consisting of 10mM Acetate (pH=4.0) containing 6M urea. A gradient 0 - 300mM NaCl was established in 10mM acetate (pH=4.0) containing 6M urea over the 5 - 30 min interval. After 30 mins the gradient went from 300mM to 1M NaCl in 10mM acetate in 6M urea over the next 5 min. The flagellin construct was collected at approximately 24 min. which corresponds to about 200mM NaCl. The fraction was dialyzed against PBS and purity determined on the material was established by Western blots using anti-flagellin antibody. A representative HPLC analysis and SDS-PAGE are shown in figures 8 and 9 respectively.

#### EXAMPLE 10: Preparation of meningococcal-flagellin glycoconjugate

Group C meningococcal capsular polysaccharide (GCM CPS: lot # 86 NM 01) was prepared essentially according to Bundel *et al.*, Bundel *et al.*, *J. Biol. Chem.* 259: 4797-801, 1976).

Nesleer's Bactericidal strain CII was obtained from the Walter Reed Army Institute (Washington, DC). The strain was precultured twice on sheep blood agar plates, then used for the inoculation of a liquid seed culture medium Nesleer's chemically defined medium, (NCDM) Kenney *et al.*, Bull. W.H.O.

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32 469-73, 1967). Finally, 40 l of liquid medium (NCDM) in a fermentor was inoculated with the liquid preculture. The purity of the strain was checked at each stage. After centrifugation, the supernatant was precipitated by addition of cerium to a final concentration of 0.1%, and the insoluble complex re-dissolved in cold 1 M calcium chloride ( $\text{CaCl}_2$ ) (Gotschlich *et al.*, *J. Exp. Med.* 123:1149-65, 1969). Ethanol (96%) was added to a final concentration of 25% (v/v). After 1 h, the suspension was centrifuged (1 h, 50,000 g), the supernatant was collected, and its ethanol concentration was increased to 80% (v/v). After 1h, centrifugation (20 min, 5,000 g) yielded a precipitate which was washed successively with absolute ethanol, acetone, and diethyl ether, and then dried in a vacuum desiccator over phosphorus pentoxide ( $\text{P}_2\text{O}_5$ ) to constant weight. This crude CPS was stored at -20°C.

In order to obtain a purer preparation, the CPS was then dissolved in sodium acetate buffer (1.10 dilution of a saturated solution, pH 7.0) and extracted four times with hot phenol (Westphal *et al.*, *Z. Naturforsch.* 7b:148-55, 1952). After dialysis of the combined aqueous phases against 0.1 M  $\text{CaCl}_2$ , followed by centrifugation (3-5 h, 100,000 g), a final ethanol precipitation was performed on the clear supernatant, and the resulting precipitate washed with organic solvents

and dried, as described above. The pure CPS was then stored at -20°C.

At each stage of the purification process, the CPS was analyzed for carbohydrate N-acetyl-neuraminic acid, NANA) (Svennerhold, *Biochim. Biophys. Acta* 24:504, 1957), D-acetyl (Hestrin, *J. Biol. Chem.* 180:249, 1949), and protein (260 nm detection) content, and its molecular weight checked by gel filtration.

Group C meningococcal capsular polysaccharide (GCM CPS) was simultaneously depolymerized and activated via sodium periodate ( $\text{NaIO}_4$ ) oxidation in aqueous buffer (Anderson *et al.*, *J. Immunol.* 137:1181-6, 1986; Eby *et al.*, *Pediatr. Res.* 20:308A, 1986; Anderson *et al.*, *J. Pediatr.* 111(5):644-50, 1987; Anderson, *U.S. Pat.* 4,762,713; 1988). The reaction was monitored by high performance gel permeation chromatography (HGPC) in aqueous eluent, using ultraviolet (UV) and refractive index (RI) detection. The reaction was stopped and the activated oligosaccharides (GCM OS) were desalted by low pressure gel permeation (GPC) in water, and then lyophilized. A solution was then prepared in water and subsequently frozen for temporary storage. GCM OS and flagellin pCB12-10-6 were mixed in aqueous neutral buffer and the conjugation was initiated by addition of sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ ) (Anderson, U.S. Patent 4,762,713, 1988; U.S. Patent 4,673,574, 1987; U.S. Patent 4,761,283, 1988). The reaction was carried out for 5 days, while being

monitored by HGPC. It was finally stopped by dialysis/concentration on centrifugal microconcentrators. The final preparation was stored in the cold, in the presence of thimerosal to prevent bacterial growth. The resulting glycoconjugate not only provides a mechanism to present the expressed VR1 and VR2 meningococcal epitopes to the immune system but also serves as a carrier molecule for the presentation of a meningococcal oligosaccharide.

In preparation of the conjugate, the following conditions were employed. Purified flagellin pCB12-10-6 was dissolved in 15% sucrose (3.5 mg/ml) and then stored at -20°C. GCM CPS (9.7 mg; final concentration: 5 mg/ml) was oxidized by 100 mM  $\text{NaIO}_4$  in 0.05 M sodium phosphate buffer (pH 6.2 - 6.5) at RT, in the dark, with agitation. Aliquots (100  $\mu\text{l}$ ) were withdrawn at regular intervals, the reaction stopped by addition of ethylene glycol (10  $\mu\text{l}$ ), and analysis was performed by HGPC on Waters (Milford, MA) Ultrahydrogel  $\text{TM}$  250 + 120 (2 columns coupled; 2 x 300 mm x 7.8 mm) in 0.2 M phosphate-saline buffer (PBS; 0.2 M sodium phosphate, 0.9% NaCl, pH 7.8), at a flow rate of 0.8 ml/min, using UV (206 nm) and RI detection. After 2 h 30 min, the reaction was stopped by addition of ethylene glycol (1/10 of the reaction volume), and the GCM OS were desalted by GPC on Bio-Rad (Richmond, CA) Bio-Gel<sup>®</sup> P-2 (200-400 mesh, 30 cm x 1.5 cm) in water, at about 18 ml/h. Fractions were collected (1.2 ml) and analyzed for the presence of NANA the carbohydrate N-acetyl-



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neuraminic acid (NANA) (Barry *et al.*, *J. Gen. Microbiol.* 29:335-32, 1962) and aldehydes (Porro *et al.*, *Anal. Biochem.* 118:301-306, 1983). Positive fractions were pooled and lyophilized. Desalted GCM OS (4.7 mg) were then dissolved in water (10 mg/ml) and frozen at -20°C.

Both GCM OS and PCB12-10-6 solutions were analyzed by HPLC (UV at 206 and 280 nm respectively) before being frozen, and prior to the conjugation. No degradation occurred during storage, as ascertained by the exact similarity of the elution profiles.

GCM OS (2 mg; final concentration: 2.6 mg/ml) and flagellin PCB12-10-6 (2.3 mg; final concentration: 3 mg/ml) were mixed in a polypropylene tube in 0.6 M sodium phosphate buffer (pH 7.0), and NaBH<sub>4</sub>CN was added (12  $\mu$ moles) to initiate the conjugation (Anderson, U.S. Patent 4,762,713, 1988; U.S. Patent 4,673,374; U.S. Patent 4,761,283). The reaction mixture was left one day at RT, then 4 days at 35°C, without agitation. The reaction was monitored by HPLC (UV at 280 nm) at different stages, and finally stopped by dialysis/concentration on microconcentrators. The final preparation was analyzed for NANA (Barry *et al.*, *J. Gen. Microbiol.* 29:335-353, 1962) (0.09 mg at 0.12 mg/ml) and protein (Lowry *et al.*, *J. Biol. Chem.* 193:265-273, 1951) (1.12 mg; 1.45 mg/ml) content. It was then stored at 4°C in the presence of thimerosal (0.01%, w/v) to prevent bacterial growth.

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The conjugate preparation was also checked by SDS-PAGE (silver nitrate stain) and Western blot analyses. Several high molecular weight bands appeared on the gel above the pure PCB12-10-6 band and near the stacking wall, the latter being an evidence that cross-linking occurred during conjugation. Western blot analyses showed that each band was reactive with the antisera used (anti-GCM, -VRL, and -VR2), proving covalency of the conjugate bonds.

#### EXAMPLE 11: Conjugation of Noninfectious peptides to GCM and bovine serum albumin

Peptides designated as H20 and H21 were produced on an ABI model-peptide synthesizer by solid phase synthesis using the CBOC chemistry were coupled to GCM<sub>197</sub> (prepared as described by Anderson, U.S. Patent No. 4,762,713) using a bifunctional crosslinking agent, sulfo succinimidyl (6-iodoacetyl) amino benzoate (Sulfo SIAB; purchased from Pierce) following the modification of a published procedure (Waltman, J.K. *et al.*, (1983) *Bio Techniques* 1, 148-152). Briefly, GCM<sub>197</sub> was activated by sulfo SIAB resulting in the formation of an amide bond between SIAB and amino groups of GCM<sub>197</sub>. After the removal of unreacted crosslinker from the activated GCM<sub>197</sub> by gel filtration, peptide (H20 or H21) containing linking spacer (represented in underlined letters) with carboxy terminal cysteine residue was

mixed with activated CRM and incubated at room temperature for 2-4 hours. Following the reaction, the conjugated material was dialyzed extensively against PBS at 4 C.

The sequence of M20 peptide (VR2 epitope) is as follows:

H-Tyr-Thr-Thr-Lys-Asp-Thr-Asn-Asn-Asn-Leu-Thr-Leu-Val-Lys-Ala-Gly-Ala-Cys-OR

The sequence of M21 (VR1 epitope) peptide is:

H-Ala-Gln-Ala-Ala-Asn-Gly-Gly-Ala-Ser-Gly-Gln-Val-Lys-Ala-Gly-Ala-Cys-OR.

Conjugated materials were subjected to SDS

PAGE, transferred to PVDF membranes (Immobilon, Millipore) and reacted with specific monoclonals which recognize VR1 and VR2 epitopes. Figure 10a and 10b show the western blot analysis of M20 and M21 CRM<sub>197</sub> conjugates, against a pool of VR1 and VR2 specific monoclonals (Adam T, C2-D12-8 (Pl.7), MS5-G11-G (Pl.16) and M14-G11-6 (Pl.7)).

In order to assay the antibody response to M20 and M21 peptide by enzyme linked immunoassay procedure, BSA conjugates were prepared by using a different bifunctional crosslinking agent, N-Succinimidyl Bromoacetate as described by Bernatowicz and Matsueda (Anal. Biochem. 155, 95-102 (1986)).

Covalent coupling of peptide to the protein was confirmed by western blotting of electrophoresed samples as described for CRM<sub>197</sub> conjugates.

EXAMPLE 12: Retention of T cell activity by M20 and M21-CRM<sub>197</sub> conjugates

To determine whether conjugation of the VR1 and VR2 epitopes to CRM<sub>197</sub> adversely affect the T cell recognition of the CRM<sub>197</sub> protein a T cell proliferative assay was performed as previously described by Bixler and Atassi (Immunol. Commun. 12:593, 1983). Briefly, SJL/J mice were immunized with 50 µg of native CRM<sub>197</sub> emulsified in CFA. Seven days later, lymph nodes were removed, cultured in RPMI and challenged with various concentrations of proteins (0.05-100.0 µg/ml) and peptides. After 3 days incubation, cultures were pulsed with [<sup>3</sup>H]-thymidine for 16 hours and then harvested for counting.

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TABLE 3

T cell responses to meningococcal peptide-CRM197 conjugates.  
In Vitro Challenge

	Maximum observed $\mu$ g/ml	$\frac{1}{2}$ incorporation dcpm	SI
Diphtheria toxoid	5	27,510	57
CRM197	50	108,631	221
CRM197 - mock conjugate	100	116,326	236
M21-CRM197	100	162,499	370
M20-CRM197	10	69,972	163
CON A	1	34,316	70
LPS	50	61,519	126
Tetanus toxoid	10	515	2
Background (cpm)	-	494	1

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As shown in Table 3, a comparison of CRM197 with the CRM197-mock conjugate shows that the conjugation procedure by itself did not alter the T cell recognition of the protein. The T cell responses induced by the M20 and M21-CRM197 conjugates were essentially equivalent to or greater than the response elicited by CRM197 itself indicating that the recognition of the T cell epitopes on the CRM197 is not adversely affected by the peptide conjugation. The responses to the control materials Con A, LPS and Tetanus toxoid were as expected.

EXAMPLE 11: Immunogenicity of conjugate and recombinant meningococcal b vaccines

Recombinant flagellin expressing the meningococcal VR1 and/or VR2 epitopes were prepared and purified as described in Examples 7, 8 and 9. In addition, synthetic peptides representing the meningococcal epitopes VR1 and VR2 were synthesized, covalently coupled to the carrier molecule CRM197 and purified as in Example 12. Vaccines were formulated with each of these materials at protein concentrations of 10 or 100  $\mu$ g/ml for each of the components. The vaccine compositions also included aluminum phosphate at 1 mg/ml or except as noted were compounded with Freund's complete adjuvant or without supplemental material.

To evaluate immunogenicity, outbred Swiss Webster mice were immunized intramuscularly at weeks

0 and 2 with 1 or 10 µg protein/dose. Sera were collected at two week intervals, pooled for assay, and screened for antibody activity by ELISA to outer membrane complex (OMC), purified OMP (Pl.16), VPI peptide coupled to Bovine serum albumin (M21-BSA), VR2 peptide coupled to BSA (M20-BSA), wildtype flagellin, and to CRM<sub>197</sub>. The results of the ELISA performed on sera obtained at 6 weeks are shown in Table 4.

TABLE 4

Immunogenicity of recombinant or CRM197 conjugate vaccines containing the meningococcal Pl.16 OMP epitopes VPI and VR2.

DOSE µg	ELISA TITERS 4 WEEKS AFTER SECONDARY BOOST <sup>1</sup>				FLAGELLIN CRM	
	ORC	Pl.16	M21-BSA	M20-BSA		
PPX1650 (control wildtype flagellin) <sup>2</sup>						
1	<150	<100	171	100	427,781	ND
10	<150	100	154	<100	468,385	ND
PCB1-4						
1	532	4,376	4,525	ND	787,120	ND
10	2,034	12,367	17,565	ND	887,861	ND
PCB2-W						
1	150	308	ND	501	263,143	ND
10	1,350	12,190	ND	5,476	1,493,216	ND
PCB12-10-6						
1	615	3,374	4,631	824	299,889	ND
10	1,423	3,666	3,882	2,253	497,622	ND
PCB12-10-6 without aluminum phosphate						
1	409	739	505	597	139,147	ND
10	450	1,533	817	1,611	358,033	ND
M20-CRM197						
1	<150	<100	217	<100	ND	42.27
10	50	<100	150	<100	ND	95.31
M21-CRM197						
1	68	249	10,494	100	ND	17.41
10	110	311	26,807	191	ND	20.92
MIXTURE OF M20 AND M21 CONJUGATES						
1	50	100	40,000	187	ND	37.32
10	50	227	15,539	132	ND	184.27
OMP Pl.16						
1	12,630	17,714	100	764	ND	ND
10	23,178	67,565	162	3,276	ND	ND
PCB1-4 in CPA						
10	1,665	10,606	19,945	ND	1,841,852	ND
PCB2-W in CPA						
10	1,157	6,869	ND	17,749	1,217,063	ND

<sup>1</sup> All pre-bleed values at or below the lower limit of assay of 1/100 dilution.  
<sup>2</sup> All vaccines were formulated with 1 mg/ml aluminum phosphate except as noted.

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Alternatively, the various vaccines were evaluated for immunogenicity in 6-8 week old NIH outbred mice. The mice were immunized with 100 µg (total protein)/dose subcutaneously on week 0 and 4 with vaccine and sera was collected on week 6. The sera were evaluated in an ELISA assay and using antigens as described in Example 6. Bactericidal activity was measured as in Example 6. The results are found in Table 5.

TABLE 5  
ELISA (titer > 0.5 OD)

Vaccine	ODC	Class 1 ODC	Synth. Peptide	Bactericidal Test
<u>FLAGELLIN</u>				
PI-50	-	-	-	<1:64
PCB12-10-6	-	1:900	-	<1:64
PCB2-W	-	1:300	1:100	<1:64
PCB1-4	1:300 (.25)	1:2700	-	<1:64
CM197	-	-	-	<1:64
H2O-CM197	1:00	1:8100	-	<1:64
H21-CM197	1:300 (.125)	1:8100	-	<1:64

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The recombinant flagellins containing either a VR1, VR2 or a cassette of both VR1 and VR2 were effective in eliciting an antibody response which was cross-reactive to the purified P1.16 and to a lesser extent to OMC. Sera from animals immunized with 10 µg of either PCB1-4 or PCB2-W induced antibodies which bound to their respective peptide-BSA conjugates as well as across reacted with the P1.16 and OMC. Similar results were obtained with the constructed PCB12-10-6 which contains both meningococcal epitopes. In addition, each construction induced significant anti-flagellin titers as well. In contrast, the control wildtype flagellin only induced an antibody response to flagellin itself. Sera collected prior to immunization showed no pre-existing response to the materials being evaluated.

The data also demonstrates the benefits of formulating the recombinant flagellins with alum or other adjuvants such as CFA. The construction PCB12-10-6 was formulated with and without the addition of aluminum phosphate. As shown in table 2, PCB12-10-6 alone was capable of inducing an antibody response which react to the peptide conjugates as well as to the purified P1.16 as well as to OMC. In comparison, the same construction when formulated with alum was able to elicit greater antibody response at an equivalent dose. Similarly, the recombinant flagellins PCB1-4 and PCB2-W were also formulated with CFA. Again, equivalent or

higher antibody titers were observed in the presence of CFA.

The results of the immunogenicity studies with the meningococcal VR1 and VR2 conjugates are also shown in Table 4. Both the M20 and the M21-CRM<sub>197</sub> conjugates as well as a mixture containing equal amounts of both conjugates were capable of inducing an anti-CRM<sub>197</sub> response as well as an anti-class I OMP response.

These preliminary data indicate a Class I OMP variable region epitopes either chemically conjugated to a carrier or genetically fused to a carrier elicit an immune response. New epitope-carrier conjugates can be made using standard techniques to enhance the immune response to the vaccine, for example, use of 1) larger epitopes, 2) peptides with multiple epitope repeats and/or 3) different carriers.

#### EXAMPLE 14: Preparation of Meningococcal-human serum albumin glycoconjugate

GCM CPS was depolymerized by acid hydrolysis and GCM OS obtained were subsequently activated via NaIO<sub>4</sub> oxidation in aqueous buffer. The reactions were monitored by HPLC in aqueous eluent, using UV and RI detection. The reactions were each followed by GPC desalting in water. GCM OS and human albumin (HA) were mixed and conjugated essentially as described in Example 10 for the meningococcal-

flagellin glycoconjugate. The final preparation was stored in the cold, in the presence of thimerosal to prevent bacterial growth.

In preparation of the conjugate, the following experimental conditions were employed. Human albumin (HA; Sigma<sup>R</sup>, St. Louis, MO) was dissolved in 1% sucrose (10 mg/ml) and then stored at -20°C. GCM CPS (lot # 86 EH 01; 106 mg; final concentration: 10 mg/ml) was hydrolyzed in 0.1 N HCl at 50°C with agitation. Aliquots (25 µl) were withdrawn at regular intervals, the reaction stopped by addition of sodium hydroxide (NaOH) and analysis was performed by HPLC as described. After 3 h 40 min., the reaction was stopped by addition of NaOH, and the GCM OS were desalted by GPC. Fractions were collected (1.2 ml) and analyzed as described before. Positive fractions were pooled and lyophilized. Desalted GCM OS (89 mg) were then stored at -20°C. Activated OS were prepared by oxidation of GCM OS (11.8 mg; final concentration: 5mg/ml) with 2 mM NaIO<sub>4</sub> in 0.03 M sodium phosphate buffer (pH 6.2-6.5) at RT, in the dark, with agitation. The reaction was stopped after 30 min by addition of ethylene glycol. HPLC analyses showed no degradation of the molecular weight of the OS during activation. Desalting and colorimetric analyses were then performed as described above. The resulting activated GCM OS (8.8 mg) were dissolved in water (10 mg/ml) and frozen at -20°C.

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Both GCM 08 and HA solutions were analyzed by HPLC (UV at 206 and 280 nm respectively) before being frozen, and prior to the conjugation. No degradation occurred during storage, as ascertained by the exact similarity of the elution profiles.

GCM 08 (6 mg; final concentration: 2.5 mg/ml) and HA (12 mg; final concentration: 5mg/ml) were mixed in a polypropylene tube in 0.4 M sodium phosphate buffer (pH 7.0), and NaH<sub>2</sub>CR was added (60  $\mu$ moles) to initiate the conjugation (Anderson, U.S. Patent 4,762,713, 1988; U.S. Patent 4,673,576, 1987; U.S. Patent 4,761,283, 1988). The reaction mixture was left one day at RT, then 4 days 15 35°C, without agitation. The reaction was monitored by HPLC (UV at 280 nm) at different stages, and finally stopped by dialysis/concentration on microconcentrators. The final preparation was analyzed for RNA (Seiry et al., *J. Gen. Microbiol.*, 29735-51, 1962) (2.07 mg at 0.86 mg/ml) and protein (Lowry et al., *J. Biol. Chem.*, 19265-75, 1951) (9.51 mg at 3.96 mg/ml) content. It was then stored at 4°C in the presence of chloroform (0.01%, w/v) to prevent bacterial growth.

The conjugate preparation was also checked by SDS-PAGE (silver nitrate stain) and Western blot analyses. A diffuse band appeared on the gel which covered a significantly wider molecular weight range than the pure HA. Western blot analyses showed that this band was reactive with the antiserum used

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(anti-GCM), proving covalency of the conjugate bonds.

**EXAMPLE 13: Immunogenicity of Meningococcal oligosaccharide-recombinant flagellin vaccines**

A meningococcal oligosaccharide-recombinant flagellin vaccine was prepared as described above and formulated at 100  $\mu$ g protein/ml. Vaccine compositions were also prepared which contained aluminum phosphate (1 mg/ml) or complete Freund's adjuvant in addition to the glycoconjugate.

To evaluate the immunogenicity, outbred Swiss Webster mice were immunized intramuscularly with 10  $\mu$ g protein at week 0 and 2. Sera were collected at weeks 0, 2 and then weekly intervals thereafter, to 6 weeks. After collection, pooled sera samples were assayed for antibody activity by ELISA to meningococcal C oligosaccharide conjugate to human serum albumin, OMC, pI 16, CBI and CB2-HSA conjugates and flagellin.

The MenC-CB12-10-6 glycoconjugate was effective at eliciting an immune response which was reactive with both the oligosaccharide and the meningococcal B OMP epitopes expressed in the recombinant flagellin. As shown in Table 5b, as little as three weeks into the study, mice immunized with 1  $\mu$ g of MenC-CB12-10-6 conjugate in complete Freund's adjuvant had detectable antibody to MenC-HSA. OMP

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and to both the CB1 and CB2 epitopes. Further, all of the MenC-CB12-10-6 preparations, regardless of adjuvant, elicited antibody responses to MenC-HSA which were greater than the response observed following immunization with MenC-CRM197.

Table 5B. Immunogenicity of Meningococcal C recombinant flagellin vaccine one week after secondary immunization.

IMMUNOGEN	Dose	MenC-HSA	ELISA TITERS <sup>1</sup>			
			OMP	CB1-BSA	CB2-BSA	FLAGELLIN
MenC-CB12-10-6	CFA	10	24,530	608	5,240	432
		1	5,069	5,614	5,375	541,467
						12,685
						526,593
MenC-CRM197	alum <sup>2</sup>	10	11,845	253	835	673
		1	4,415	136	242	472,766
						244
						214,263
OMP (Pl.16)	None	10	11,497	920	626	2,382
		1	4,920	483	1,123	233,307
						1,210
						135,625
MenC-CRM197	alum	10	4,905	ND	ND	ND
		1	8,505	ND	ND	ND
						ND
						ND
OMP (Pl.16)	alum	10	ND	12,907	<100	<100
		1	ND	10,405	<100	3,377
						ND
						ND

<sup>1</sup> Titers for initial prebleed samples (week 0) samples were <100.

<sup>2</sup> Aluminum phosphate was used as adjuvant at 1 mg/ml.

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# EXAMPLE 15: T-cell epitopes of Class I OMP and their identification

An effective vaccine must contain one or more T-cell epitopes. T-cell epitopes within a protein can be predicted as described by Margalit et al., J. Immunol. 138:2213, (1987) or Rothbard and Taylor, EMBO J. 7:93, (1988). These predictive methods were applied to the amino acid sequence of the Class I OMP of *N. meningitidis* strains Pl.7.16, Pl.16 and Pl.15. The segments of the sequence containing potential T cell epitopes identified by these methods are shown in Tables 6 and 7. The predicted peptides were synthesized by standard Fmoc procedures, purified by standard methods and were identified as shown in Table 8.

To determine which of the predicted peptides actually contain T cell epitopes, their capacity to stimulate human peripheral blood lymphocytes (PBL) was tested by lymphocyte proliferative assay. Briefly, peripheral blood was collected from HLA typed normal volunteers or from volunteers who were previously immunized with MPC-2 (Poolman, J.T. et al., Antonie van Leeuwenhoek, 53:413-419, 1987) which contained Pl.16, 15, Class 4 OMP and Group C polysaccharide. Lymphocytes were isolated from the peripheral blood by isolation on Ficoll-Hypaque (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and cultured at  $1 \times 10^5$  cells/well in supplemented RPMI 1640 (Gibco Laboratories, Paisley, Scotland)



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containing 10% heat-inactivated pooled human AB serum. Cultures were challenged with various concentrations of the predicted T cell epitopes (0.05 - 10  $\mu$ g/ml). After 48-72 hours challenge, the cultures were incubated for six days and then pulsed (18 hours) with 0.5  $\mu$ Ci of [ $^3$ H]-thymidine. Cultures were then harvested and counted by liquid scintillation. Data are expressed as stimulation indices which were calculated as a ratio of the CPM obtained in the presence of antigen to the CPM obtained in the absence of antigen.

As shown in Table 9, 10 of the 16 predicted peptides showed some capacity to stimulate T-cells. These include the peptides identified at 16-36, 47-59, 78-90, 103-121, 124-137, 151-158, 176-185, 223-245, 276-291 and 304-322. In some instances, peptides stimulated a response in both immunized as well as non-immune individuals. The response in the non-immune individuals may be attributed to a previous asymptomatic infection.

In the case of the T cell epitope identified as region 176-185, enhancement of the T cell response was observed following addition of the monoclonal antibody MNSC11G (Pl.16). Briefly, PBL were challenged *in vitro* with a synthetic peptide containing the region 173-185 or with this peptide mixed with varying dilutions of MNSC11G. As shown in Table 10, enhancement of the T cell response was observed following addition of MNSC11G indicating that monoclonal antibody recognized a B cell epitope

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within the region 176-185 and facilitates the presentation of the peptide to the immune system. Thus, it was established that the T and B cell epitopes either coincide or are found on contiguous sequences within the Class I OMP.

In several cases, T cell lines and clones were established from individuals responding to various peptides. Briefly, T cell lines were obtained by culturing isolated lymphocytes in 24 well plates at  $1 \times 10^6$  cells/ml. The culture medium, supplemented RPMI-1640 with 10% human serum, also contained 12 U/ml recombinant IL-2 (Boehringer). In addition,  $5 \times 10^4$  homologous, irradiated ( $3,000R$ ) antigen presenting cells (APC) were also added to each well. In some cases, APC were obtained from HLA compatible donors. From the lines, T cell clones were isolated by limiting dilution at a frequency of 0.5 cells/well. Clones were maintained by bi-weekly stimulation with antigen in the presence of irradiated APC and IL-2 (2 U/ml). Clones were tested by lymphocyte proliferation assay essentially as described above except that clones were cultured at  $1 \times 10^4$  cells/well in the presence of irradiated APC.

Clones obtained as described were challenged *in vitro* with OMP from 7 different strains of meningococci. As shown in Table 11, the clones recognized a T cell epitope or epitopes common to the seven OMPs examined. Although the reactivity of these clones to the various peptides remains to be

determined, the data, nevertheless, does indicate the commonality of T cell epitopes among the various strains. Now that these clones have been established and identified their peptide reactivity will indicate T-cell epitopes for vaccine use.

Table 6. ANALYSIS OF THE SEQUENCE OF N. MENINGITIDIS 21.16 OMP FOR THE PRESENCE OF AMPHIPATHIC  $\alpha$ -HELICES ACCORDING TO THE METHOD OF MARGALIT ET AL. (J. IMMUNOL. 138:2113, 1987)

P	MID POINTS OF BLOCKS		ANGLES	AS
	47-50	85-105		
K	69-74	105-135	16.0	9.4
	79-88	90-120	23.0	16.0
	127-135	100-120	22.4	23.0
	199-202	90-120	8.4	22.4
*	208-211	85-95	8.7	8.4
	260-263	90-125	6.8	8.7
	265-269	90-120	11.3	6.8
	274-277	105-120	9.8	11.3
P	297-300	100-135	9.1	9.8
	320-324	80-100	10.9	9.1
	338-342	105-135	12.3	10.9
	346-351	80-115	11.9	12.3
*	376-379	85-120	9.5	11.9
				9.5

Table 7. PRESENCE OF MOTIFS (UNDERLINED REGIONS) REPRESENTING POTENTIAL T CELL EPITOPES WITHIN THE SEQUENCES OF N. MENINGITIDIS 21.16 OMP AS DETECTED BY THE METHOD OF ROTHBARD AND TAYLOR (EMBO J 7:93, 1988).

ARRKLTALVLSALPLAAADVSLYGEIKAGVSGAMI  
QAQLTEQFQVTHGVQGNQVEVTRAKSRIRTKISDPG  
SPIGFQSSDLOEGLAAVHQLSQDVSVAQQAASQWG  
HRESPIGLAGSPGTLEAGRYANQPDASQAINPWS  
NMVVASOLGIFRRRDDMPVSVRYDSPEPSQSVQ  
FVPAQNSKSAVYKPAVYTKDTMNLTLVPAVVGKPS  
DVVYAGLNYKNGGFAQNYAFKIASANVOGNAFELP  
LIGSATSDAKQTDPLENQVRLTGOYRGGGLMLA  
LAAQLDLSNGDKAKTKNSTTEIAATASYRFGHVP  
RISYANGFDLIEROKKGMTSYDQIIAQVDYDFSR  
TSAIVSGAWLERNTQIQNTQINAAASVGLRKEP

Table 8. SUMMARY OF PREDICTED T CELL EPITOPES SYNTHESIZED.

RESIDUE NO.	SEQUENCE
1. 16-34	NIGALTEQPOVYNGVGN
2. 47-59	TKISDFGFIQFR
3. 57-71	GFKGSEDLGEGKAY
4. 70-90	VSVAGGDAQGCH
5. 103-121	TLRAGRVANQFDASQAIN
6. 124-137	DSNNDAVSEDLGIFK
7. 151-158	GGFSGFSG
8. 176-185	YTKDTNNML
9. 190-202	AVVGRPSDYTYA
10. 215-220	YAFKVAHNVORH
11. 223-245	ANYQANAFELFLIGSATSDKAO
12. 241-261	DEAKOTDPLNNHVAHLTOGY
13. 276-291	LSGNODKATKSTTTE
14. 304-322	VPLISTYAGFDLIERGKGO
15. 317-329	ENCKRGENTSYDO
16. 352-366	KNNTOIGNYTOINAA

Table 9. SUMMARY OF LYMPHOCYTE RESPONSES TO NEUROCOCCAL ANTIGENIC PEPTIDES IN HLA TYPED VOLUNTEERS.

VOLUME/MLA TYPE	RESPONSE TO SYNTHETIC PEPTIDE															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. DR4, W10, W53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2. DR1, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. DR1, W11, W53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4. DR1, W6, W13, W18, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5. DR1, W11, W7, W52, W53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6. DR1, W11, W10, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7. DR1, W6, W53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8. DR1, W13, W53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9. DR1, W13, W53	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10. NOT TYPED	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11. NOT TYPED	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28. DR1, W11, W52	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

The responses were scored as follows: -, 0; 1, 1; 2, 2; 3, 3; 4, 4; 5, 5; 6, 6; 7, 7; 8, 8; 9, 9; 10, 10; 11, 11; 12, 12; 13, 13; 14, 14; 15, 15; 16, 16.

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Table 10. PRESENTATION OF A SYNTHETIC PEPTIDE TO PERIPHERAL BLOOD LYMPHOCYTES IS ENHANCED BY A MONOCLONAL ANTIBODY RECOGNIZING REGION 179-184 OF MENINGOCOCCAL CLASS I ORP.

IN VITRO CHALLENGE	CPM
GGYTKDTNNL	3,017
GGYTKDTNNL* + MNSC11G (1:200)	22,836
GGYTKDTNNL + MNSC11G (1:1000)	12,608
MEDIA	330

\*Underline region indicates sequence recognized by monoclonal antibody MNSC11G.

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Table 11. RECOGNITION OF ORP FROM DIFFERENT MENINGOCOCCAL STRAINS BY HUMAN T-CELL CLONES

STRAIN SUBTYPE	RESPONSE OF HUMAN T CELL CLONES (CPM X 10 <sup>-3</sup> )							
	5-3	5-7	5-9	5-11	5-13	5-14	5-15	5-15
H44-76 P1.16	6.0	1.2	6.8	2.6	2.3	9.5	1.5	
SWISS 4 P1.15	4.9	1.0	10.1	6.9	3.6	10.5	1.4	
395 P1.9	5.2	1.5	4.8	1.5	6.1	13.1	1.4	
2996 P1.2	5.4	1.0	3.7	2.3	3.4	11.8	1.0	
M990 P1.6	3.6	0.4	3.5	2.5	0.9	4.7	0.6	
187 P1.1	4.4	0.7	4.5	3.1	1.6	6.2	1.4	
6557 P1.17	3.7	2.0	8.2	4.2	1.7	6.3	0.8	
MEDIA --	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

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EXAMPLE 17:

Construction of Protein Model for  
Membrane Topology of Class I OMP and  
Comparison to Other Pathogenic Gram  
Negative Polym Proteins for Vaccine  
Development.

A model was constructed using the principles recognized for the structure of several Escherichia coli outer membrane proteins (Vogel, H. et al. (1986) supra Ferenci, T. et al. (1988) supra; and Tomassen, J. (1988) supra). The central assumption is that protein segments spanning the outer membrane form beta-sheets. Specifically, in the case of Class I protein, the division in exposed and trans-membrane segments was arrived at in the following way:

1. A comparison of the amino acid sequence of Class I protein (subtype Pl.16) with those of the Gonococcal PIA and PII proteins (Carbonetti, R.H. et al. (1987) PNAS 84:9084; Carbonetti, R.H. et al. (1988) PNAS 85:6841; and Oroschlich, E.C. et al. (1987) PNAS 84:8135) reveals 34% identity. In the model, the variable sequences form the surface-exposed parts, whereas the conserved regions are placed mostly in the outer membrane and periplasm. Thus, the latter two areas consist for 58% of residues that are conserved among all proteins,

2. The hydrophilic maxima observed in a hydrophathy profile (Kyte, J. et al. (1982) J. Mol. Biol. 157:105) to correspond to exposed regions.
3. The transmembrane segments should preferentially be able to form amphipathic beta-strands of 9-12 residues, with at least one side consisting entirely of hydrophobic residues. These conditions are met in 12 of the 16 membrane-spanning segments.
4. The number of residues at the periplasmic side is minimized.

Figure 11 shows the model for the folding of Class I protein in the outer membrane. The sequence shown is for subtype Pl.16. The top part of the Figure shows the surface-exposed regions, whereas the central part indicates the presumed trans-membrane segments, whose length is set at ten. Amino acids are shown alternating where they can form an amphipathic beta-strand. This model contains eight surface loops, whereby the first and the fourth loop contain the type-specific and protective variable region epitopes. These epitopes, as has been shown when formulated into a vaccine, can elicit a protective immune response. Loop 5 is constant and has been shown to elicit cross-reactive antibodies to other OMPs and is useful for vaccine formulation.



LOOP 4

-FRAND KFY	SVRSDPEISGSGSVQV	PAORS
GAQDER RYI	SVRDSERFAGSGSVQV	PDORS
PEREV---	SVRDSERFAGSVQV	PDORS
TIGVESREI	SVRDSYFAGSGSVQV	VYRDN
*****	*****	*****
KAATPAATYEDTNNLTLVZAVGKPS	DYVYA	
GS-----	NG EREV	
GR-----	NBS RYRA	
ND-----	VDKTEKSSR RYRA	

LOOP 5

GLNYNGOFAGNATKTA	RENAVGBNAPELFLIG
*****	*****
GLNYNGOFAGNATKTA	RYGEGTKN-----
*****	*****
GPYNGSGFVQVAGFYK	RSTYTBAPFL
*****	*****
GLNYNGOFAGNATKTA	GSFATVADLNTD-----
*****	*****
BAISDEALGTDFLNR	QVRLTQGYBEGGNTALA
*****	*****
DDGTYSIBSFVENT	QVRLVQGYDNALVYVA
*****	*****
QVRLVQGYDHATVYVA	*****
*****	*****
RYAVNTANABFVNDY	QVRLVQGYDHATVYVA
*****	*****

LOOP 6

AGLD LBS---	NDKAKTENSTTE	LAATASYPORA
*****	*****	*****
AGQD	DAKYGALSGSHSGT	VAAZALYPRGV
*****	*****	*****
VQDQ	DAKTEARD-SHSGT	VAAZALYPRGV
*****	*****	*****
QY	YEAARNEYSTOKESQY	VAAZALYPRGV
*****	*****	*****

LOOP 7

VYRISYASNDLI	REKAKTENSTYQ	ILASTYDQ
*****	*****	*****
TPRVSYASNDLI	VDANEDMT-YDQ	VYVGLBYD
*****	*****	*****
TPRVSYASNDLI	VDANEDMT-YDQ	VYVGLBYD
*****	*****	*****
TPRVSYASNDLI	VDANEDMT-YDQ	VYVGLBYD
*****	*****	*****

LOOP 8

PSKRSALYBAGT	KNYTOIGRYQIN	AAVGLABRY
*****	*****	*****
PSKRSALYBAGT	QGGKADLY-SY	AAVGLABRY
*****	*****	*****
PSKRSALYBAGT	QGGKADLY-SY	AAVGLABRY
*****	*****	*****
PSKRSALYBAGT	QGGKADLY-SY	AAVGLABRY
*****	*****	*****

Structural similarities are indicated with transmembrane and surface loop regions. With the information now available for Class I OMP and information based on surface loop size, location, intraspecies amino acid homology or heterology of the loop regions of the particular porin protein, predictions of epitopes for incorporation into vaccines for other pathogenic gram negative bacteria including *N. meningitidis* are possible. Using the same methods employed for Class I OMP, these epitopes can be evaluated for vaccine purposes.

#### Deposit of Microorganisms

The *N. meningitidis* strain H4476 (B:15:P1.7.16) was deposited on December 11, 1989 in the Central Bureau voor Schimmelculturen (CBS), Baarn, The Netherlands and has deposit number CBS 635.89. The *N. meningitidis* Class 2/3 OMP deficient mutant H11-5 was deposited on December 11, 1989 in the CBS, Baarn, The Netherlands and has deposit number CBS 636.89.

#### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

#### CLAIMS

1. Vaccine effective against meningococcal disease, comprising an effective amount of an outer-membrane vesicle isolated from expressing homologous and/or heterologous or at least one meningococcal class I outer-membrane protein or a fragment or oligopeptide containing an epitope thereof.
2. A vaccine of Claim 1, wherein the meningococcal Class I outer-membrane protein originates from a mutant meningococcal strain which is negative for Class 2/3 outer membrane protein.
3. A vaccine of Claim 1, wherein the Class I outer-membrane protein, fragment or oligopeptide is produced by a microorganism containing a heterologous gene encoding the Class I outer-membrane protein, fragment or oligopeptide.
4. A vaccine of Claim 1, wherein the Class I outer-membrane protein, fragment or oligopeptide is derived from a group A,B,C,W-135 or Y meningococcus.
5. A vaccine of Claims 1, wherein the Class I outer-membrane protein, fragment or



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- oligopeptide is derived from a group B meningococcus.
6. A vaccine of Claim 1, comprising 5-10 various group B meningococcal class I outer-membrane proteins, fragments or oligopeptides.
  7. A vaccine of Claim 1, wherein the protein fragment is obtained by cyanogen bromide treatment of a class I outer-membrane protein.
  8. A vaccine of Claim 1, wherein the fragment of class I meningococcal outer-membrane protein is obtained by proteolysis with an enzyme selected from the group consisting of endolya-G, endo-Arg-G, endoglu-G and Staphylococcus V8-protease.
  9. A vaccine of Claim 1, wherein the oligopeptide comprises at least one bactericidal antibody binding epitope of meningococcal class I outer-membrane proteins.
  10. A vaccine of Claim 1, wherein the oligopeptide comprises at least an amino acid sequence selected from the group consisting of QPQVTHGVQGM, PPSKQP, QANAGCASC, YTKDTHNHLI, YTKTHNHLI, YTKDTHNHLI, YTKTHNHLI, HFWQTPQSQP and HTRQNHNDVF.

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11. A vaccine of Claim 1, wherein the epitope is located in surface loops of meningococcal class I outer-membrane proteins in the area of amino acids 24-34 and 176-187.
12. A vaccine of Claim 1, wherein the class I outer-membrane protein, fragment or oligopeptide is conjugated to a T cell epitope via chemical coupling.
13. A vaccine of Claim 1, wherein the class I outer-membrane protein, fragment or oligopeptide contains a meningococcal A,B,C,W-135 and/or Y polysaccharide in conjugate form with the protein product.
14. A vaccine of Claim 1, further comprising the a Zwitterionogenic, cationogenic, anionogenic and/or non-ionogenic detergent.
15. A vaccine of Claim 14, wherein the detergent is selected from the group consisting of Zwittergent 2w 3-10, Zwittergent 2w 3-14, Tween-20, sodium cholate, octyl glucoside and sodium deoxycholate.
16. A vaccine of Claim 1, further comprising the an adsorbent selected from group consisting of aluminum phosphate, aluminum hydroxide and calcium phosphate.

Class I outer-membrane protein which varies in amino acid sequence among different meningococcal porin proteins.

24. Oligopeptide of Claim 23, containing at least one of the amino acid sequences selected from the group consisting of QPQVINGVQGM, PPSKSGP, QANGCAGC, YTKETNNL, YTKETNNL, YTKETNNL, YTKETNNL, YTKETNNL, HFVQTPQSQP and/or HYTRQNNTDVF.

25. Oligopeptide containing an epitope of a Class I outer-membrane protein which is conserved in amino acid sequence among different meningococcal porin proteins.

26. Oligopeptide containing a T cell epitope of a meningococcal Class I outer-membrane protein.

27. Isolated nucleic acid encoding meningococcal class I outer-membrane protein or a fragment or oligonucleotide containing an epitope thereof.

28. A method of eliciting a protective immune response against *Neisseria meningitidis*, comprising administering a vaccine composition, comprising one or more meningococcal Class I outer membrane proteins or fragments thereof and, optionally, an adjuvant in a pharmaceutically acceptable vehicle.

17. A vaccine of Claim 1, further comprising an immuno-stimulating complex (ISCOM).

18. A vaccine of Claim 1, wherein the Class I outer membrane protein, fragment or oligopeptide is contained within a liposome.

19. A vaccine of Claims 1, the Class I outer membrane protein, fragment or oligopeptide is coupled to a lipid.

20. Substantially purified fragment of class I outer membrane protein of *Neisseria meningitidis*, the fragment having a molecular weight of about 25 kD or less and containing continuous or discontinuous epitopes reactive with bactericidal antibodies against *N. meningitidis*.

21. A fragment of Claim 20, wherein the class I outer membrane protein is of the subtype Pl.7.16.

22. A fragment of Claim 21, produced by cyanogen bromide cleavage of a class I outer membrane protein of *N. meningitidis*, the fragment having a molecular weight of approximately 25 kD.

23. Oligopeptide containing a B cell epitope of a

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29. A method of Claim 28, wherein the fragment is conjugated or genetically fused to a T cell epitope, B cell epitope or carrier peptide or protein.
30. A method of Claim 29, wherein the conjugation is through a cysteine or lysine residue coupled to a terminus of the fragment.
31. A method of Claim 29, wherein the carrier is a bacterial toxin, CRM or toxoid.
32. A method of Claim 28, wherein the class I outer membrane protein is of subtype Pl.7,16.
33. A method of eliciting a protective immune response against *Neisseria meningitidis*, comprising administering a vaccine composition, comprising at least one oligopeptide containing a continuous or discontinuous epitope of a class I outer membrane protein reactive with bactericidal antibodies against *N. meningitidis* and, optionally, an adjuvant in a pharmaceutically acceptable vehicle.
34. A method of Claim 33, wherein the oligopeptide contains an amino acid sequence selected from the group consisting of QPQVNCVQGN, PPSKQF, QANCGASG, YTKDINMIL, YTKNINMIL, YTKDINMIL, YTKNINMIL, HPGQTPQSQP and/or HYRQNTDVF.

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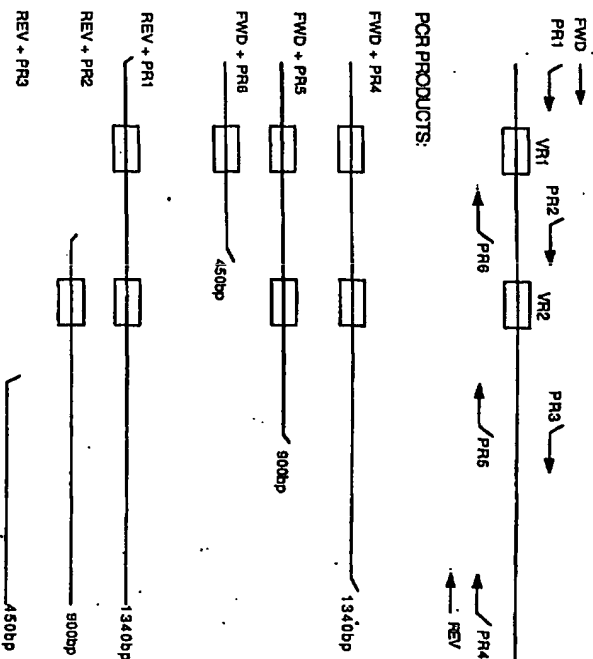
35. A method of Claim 33, wherein the proteolytic fragment is conjugated or genetically fused to a T cell epitope, B cell epitope or carrier peptide or protein.
36. A method of Claim 33, wherein the conjugation is through a cysteine or lysine residue coupled to a terminus of the fragment.
37. A method of Claim 35, wherein the carrier is a bacterial toxin, CRM or toxoid.
38. An antigenic conjugate, comprising a meningococcal class I outer-membrane protein, a fragment or oligopeptide containing a epitope thereof conjugated to a carrier protein or epitope thereof.
39. An antigenic conjugate of Claim 8, wherein the oligopeptide selected from the group consisting of QPQVNCVQGN, PPSKQF, QANCGASG, YTKDINMIL, YTKNINMIL, YTKDINMIL, YTKNINMIL, HPGQTPQSQP and/or HYRQNTDVF.
40. An antigenic conjugate of Claim 39, wherein the antigen carrier protein is a bacterial toxin, CRM or epitope thereof.
41. An antigenic conjugate of Claim 40, wherein the carrier protein is CRM<sub>197</sub>.

42. A genetic fusion peptide or protein, comprising an epitope of a meningococcal class I outer-membrane protein fused to a carrier protein, peptide or epitope thereof.
43. A genetic fusion peptide or protein of Claim 42, wherein the epitope is selected from the group consisting of QPQTVNGVQCN, PPSKSQP, QAANGCASC, YTYKDTNNLTL, YTYKNTNNLTL, YTYKDTNNL, YTYKNTNNL, HFVQTPQSQP and/or HYTRQNTDVF.
44. A fusion protein, comprising a flagellin protein having an amino acid sequence for an epitope of a meningococcal class I outer-membrane protein inserted within it.
45. The fusion protein of Claim 44, wherein the amino acid sequence is inserted within the flagellin protein at a region which is non-essential to function of the flagellin protein.
46. The fusion protein of Claim 45, wherein the amino acid sequence is inserted into the hyper-variable region of the flagellin protein.

47. A fusion protein of Claim 44, further comprising a meningococcal capsular oligo- or polysaccharide conjugated thereto.
48. A recombinant gene, encoding a fusion protein of Claims 44.
49. An infectious, recombinant microorganism capable of expressing a class I outer membrane protein of *Neisseria meningitidis*, or a fragment or oligopeptide containing a continuous or discontinuous epitope reactive with bactericidal antibodies against *N. meningitidis*.
50. A microorganism of Claim 49, wherein the oligopeptide selected from the group consisting of QPQTVNGVQCN, PPSKSQP, QAANGCASC, YTYKDTNNLTL, YTYKNTNNLTL, YTYKDTNNL, YTYKNTNNL, HFVQTPQSQP and/or HYTRQNTDVF.
51. A microorganism of Claim 49, which is a vaccinia virus, adenovirus, or cytomegalovirus.
52. A microorganism of Claim 49, which is a bacteria of the genus Salmonella.
53. A microorganism of Claim 49, wherein the epitope of the meningococcal class I outer-membrane protein is expressed as a recombinant flagellin.

54. A method of immunizing against Neisseria meningitidis comprising immunizing an individual with the microorganism of Claim 49.
55. A mutant meningococcal strain incapable of producing Class 2 and 3 outer-membrane protein.
56. The mutant meningococcal strain H115, CBS 636.89
57. A mutant meningococcal strain, incapable of producing Class 2 and 3 outer-membrane protein, transformed with a heterologous gene encoding a non-native Class 1 outer-membrane protein.
58. A method of identifying vaccine epitopes of a Gram negative porin protein, comprising:
  - a. obtaining the amino acid sequence of the porin protein;
  - b. comparing the amino acid sequence of the porin protein to the amino acid sequence of a meningococcal Class I outer-membrane protein (OMP) to predict the location, size and sequence of exposed surface loop structures of the porin protein based upon the known corresponding structures in the Class I OMP; and
  - c. identifying potential vaccine epitopes in the surface loop structures based upon the predicted location, size and sequence of the structures.

Figure 1





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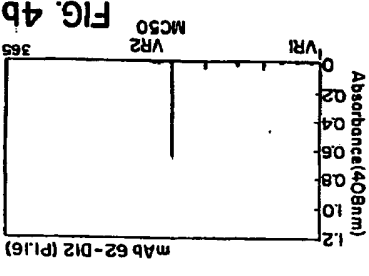
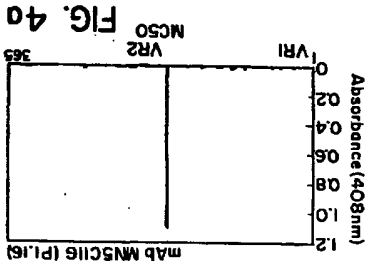
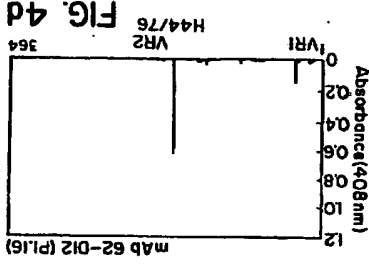
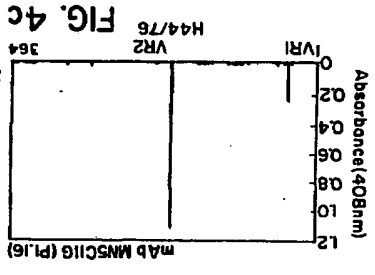
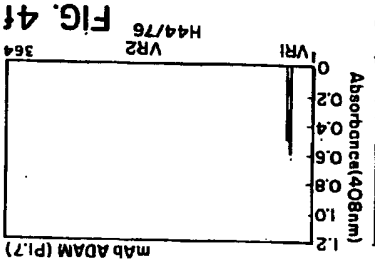
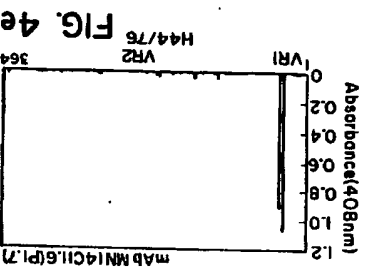
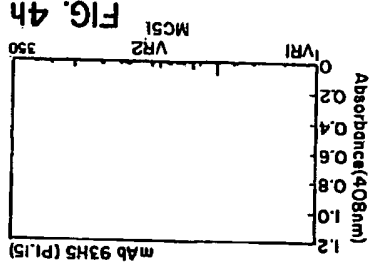
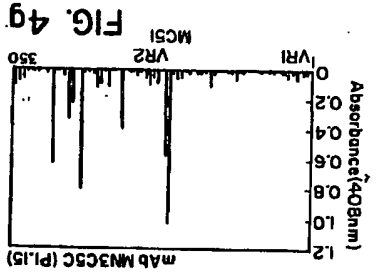
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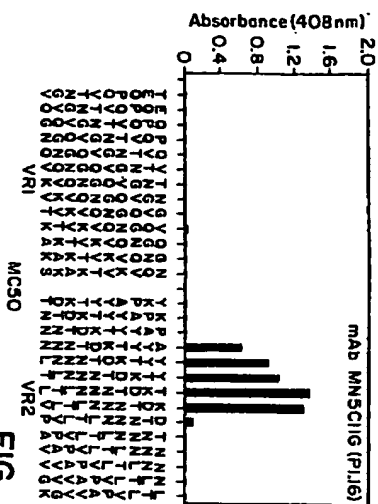


FIG. 5b

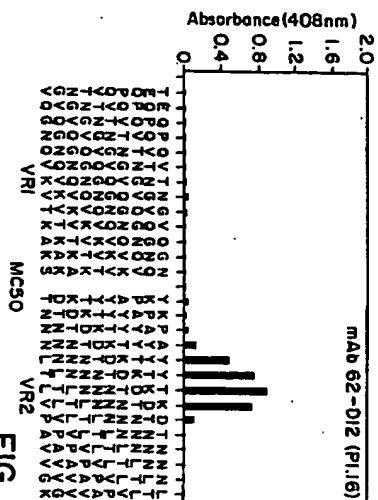


FIG. 5c

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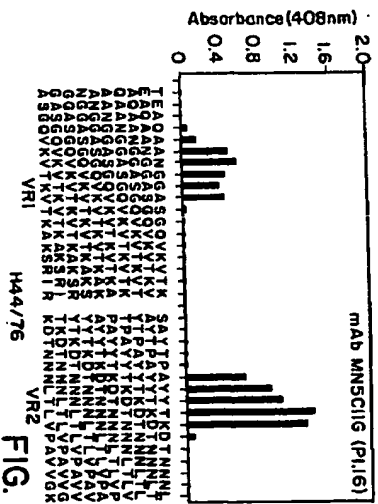


FIG. 5d

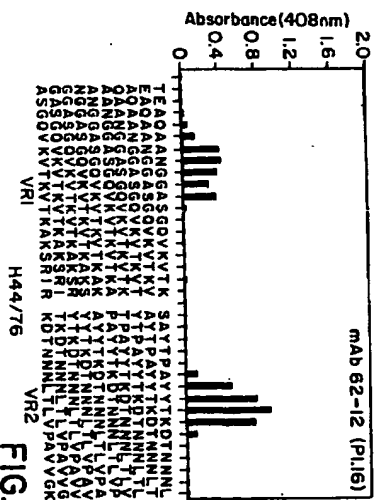


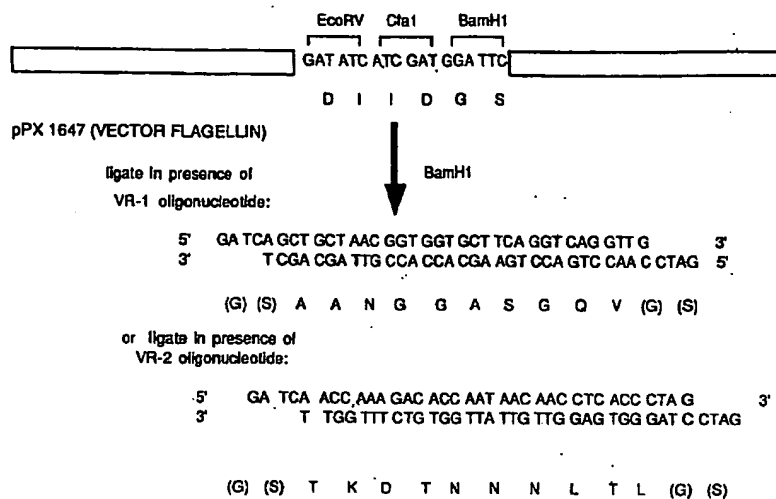
FIG. 5e

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Figure 6. HYBRID N. MENINGIDITIS FLAGELLIN CONSTRUCTIONS

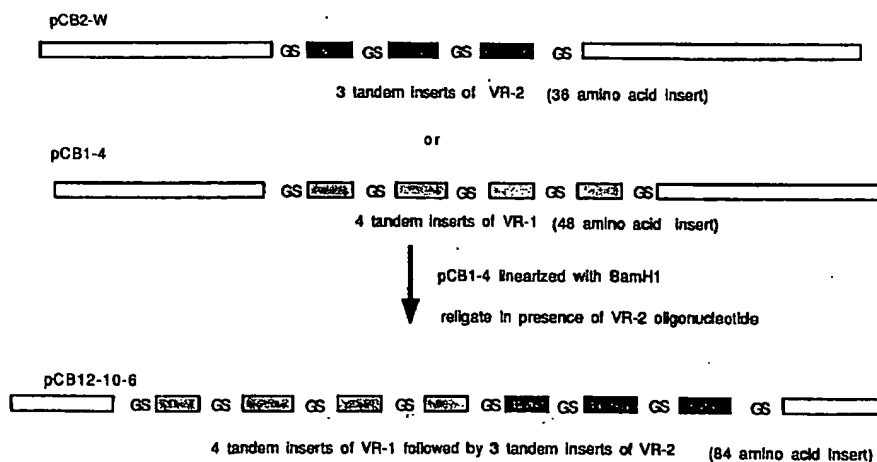


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Figure 7. HYBRID N. MENINGIDITIS FLAGELLIN STRUCTURES

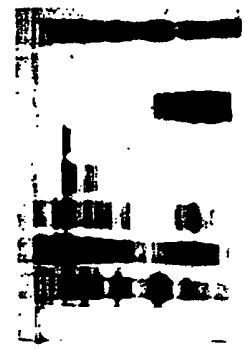


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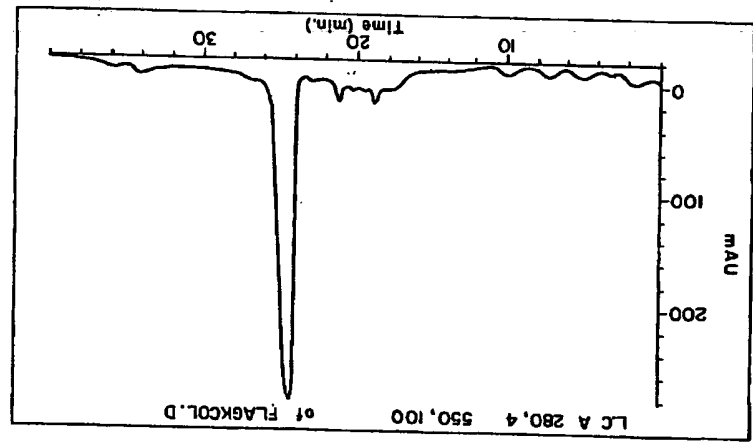
FIG.9

A representative SDS-Page of pCB12-10-6.



Lane#	Sample
1	Blank
2	High Molecular Weight Standard
3	Dialyzed pCB12-10-6, step 2
4	Fractions off peak pCB12-10-6
5	Purified pCB12-10-6 from HPLC, dialyzed in PBS, 20 µg
6	Purified pCB12-10-6 from HPLC, dialyzed in PBS, 1 µg
7	LPS - 5828
8	Blank
9	High Molecular Weight Standard
10	Blank

FIG. 8. A representative HPLC of purified pCB12-10-6 showing a single major peak



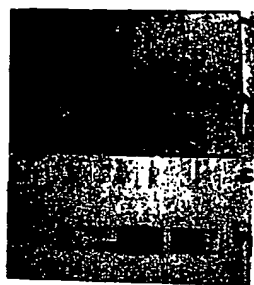
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Photographs of representative western blot analysis of CB1 and CB2 CRM197 conjugates.

**FIG. 10**

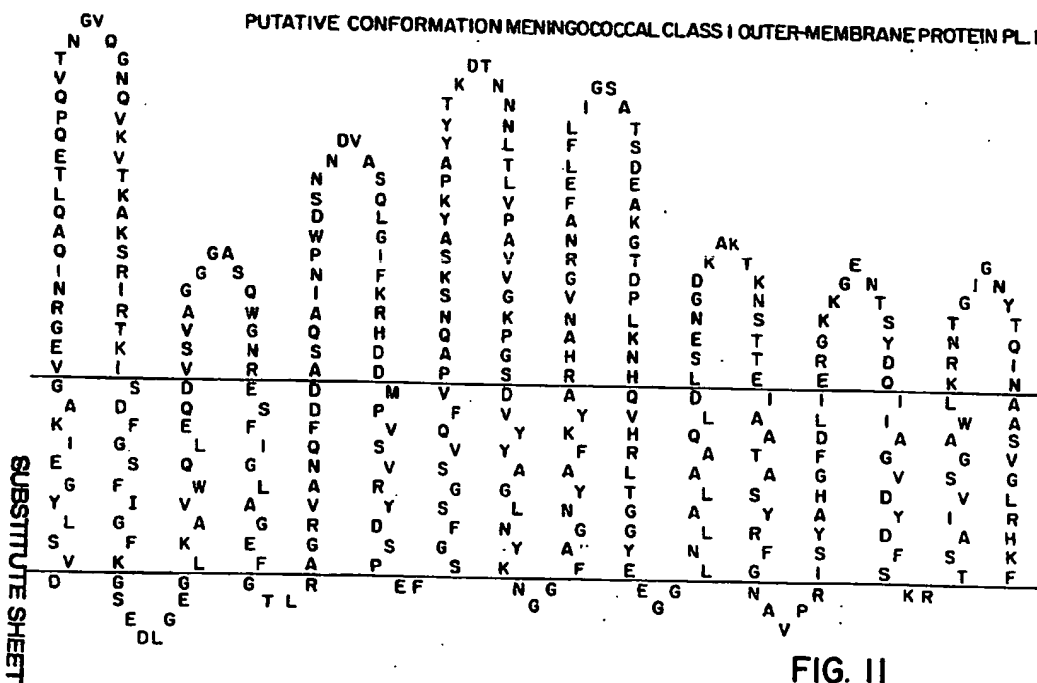


Lane#	Sample
1	Molecular Weight Standard
2	M20-CRM, 10 µg
3	M20-CRM, 20 µg
4	Molecular Weight Standard
5	M21-CRM, 1 µg

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PUTATIVE CONFORMATION MENINGOCOCCAL CLASS I OUTER-MEMBRANE PROTEIN PL 16



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FIG. 11

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